REJECTIONS UNDER 35 U.S.C. §§ 101 AND 112, FIRST PARAGRAPH

The Examiner maintains rejection of claims 25-79 under 35 U.S.C. § 101 as allegedly not being "supported by either a specific and substantial asserted utility or a well-established utility." *See*, Paper No. 24, page 2.

The Examiner further alleges:

> "[t]he instant claims are drawn to a polypeptide which has an as yet undetermined function or biological significance ... the instant specification does not disclose any information regarding functional characteristics or the biological activity of the instantly claimed protein other than it may stimulate cell proliferation and/or differentiation and may be used to treat, restenosis and inflammation."

See, Paper No. 24, page 3. Applicants respectfully disagree and traverse this assertion.

Applicants contend that the polypeptides of the invention do indeed have a determined function and biological significance. In support of this contention, Applicants respectfully direct the Examiner's attention to the teachings of Graf et al., which were previously submitted as Exhibit D on May 9, 2003, in connection with the present application. A second legible copy of Graf et al. is enclosed herewith as Exhibit D for the Examiner's convenience. As discussed in more detail below, Graf and coworkers elegantly demonstrate that polypeptides of the present invention are expressed and play a role in the regulated development of T cells in the thymus. Accordingly, Applicants believe that the Examiner has mischaracterized the polypeptides of the present invention.

Contrary to the Examiner's comments, Applicants have set forth in the specification statements that clearly and fully describe the function of Human Cytokine Polypeptide of the present invention and explain why Applicants believe the invention is useful. For example, the specification, at page 3, lines 5-8, teaches that polypeptides of the present invention may be used to treat inflammation. Preliminarily, Applicants point out that it is not necessary to identify the mechanism by which the invention operates,

rather it is necessary to assert a specific and substantial utility for the invention. Accordingly, Applicants contend that specific and substantial utilities have been disclosed in the specification as filed and that the only issue is whether any asserted utility is credible.

Inflammation

1 Inflammation requires T-cell recruitment and activation

Applicants have made the assertion that polypeptides of the present invention may be used to treat inflammation, a process which is dependent on T-cell maturation and activation. Applicants respectfully direct the Examiner's attention to the teachings of Janeway and Travers, which were previously submitted as Exhibit A on May 9, 2003, in connection with the present application. A second legible copy of Janeway and Travers is enclosed herewith as Exhibit A for the Examiner's convenience. Inflammation is a result of the inflammatory response, which occurs when a tissue is exposed to any one of a number of noxious stimuli including, for example, bacterial infections. (*See*, e.g., Exhibit A). As described by the authors, the initial inflammatory response is characterized by certain specific events including blood vessel dilation, local increase in vessel stickiness for passing immune cells, and increased vessel permeability to fluid and immune cells. The first cells involved in the inflammatory response are non-specific 'inflammatory cells' such as monocytes and neutrophils; however, later recruitment and activation of T cells may serve to sustain chronic inflammation.

Accordingly, at the time of filing of the present application, it was widely appreciated in the art that inflammation was a process involving a series of specific steps and requiring recruitment and activation of T-cells.

2 T-cells secrete cytokines required to coordinate the inflammatory response

T-cells regulate and maintain an inflammatory response by secretion of a variety of pro-inflammatory cytokines. Applicants respectfully direct the Examiner's attention to the teachings of Abbas, Lichtman and Pober, which were previously submitted as Exhibit B on May 9, 2003, in connection with the present application. A second legible copy of Abbas, Lichtman and Pober, is enclosed herewith as Exhibit B for the Examiner's convenience. T cells are recognized as playing a significant role in the maintenance of the inflammatory response, for example, through the secretion of pro-inflammatory cytokines. (*See*, e.g., Exhibit B). Abbas et al. describe "cytokines that activate inflammatory cells" including gamma interferon, lymphotoxin, interleukin-5 and migratory inhibition factor as all being produced by T cells. These cytokines serve to mobilize and/or activate numerous cells involved in the inflammatory response including, for example, mononuclear phagocytes, endothelial cells, neutrophils, eosinophils and B cells. (*See,* Table 11-3, *Id.* at page 239).

Accordingly, at the time of filing of the present application, it was widely appreciated in the art that inflammation required T-cell secretion of well-known pro-inflammatory cytokines.

3 T-cells are only produced in the thymus

T-cells are believed to develop exclusively in the thymus into adult life. Applicants respectfully direct the Examiner's attention to the teachings of Abbas, Lichtman and Pober, which were previously submitted as Exhibit C on May 9, 2003, in connection with the present application. A second legible copy of Abbas, Lichtman and Pober, is enclosed herewith as Exhibit C for the Examiner's convenience. Maturation and selection of T cells occurs in the thymus and continues into adult life. (*See*, e.g., Exhibit

C). The authors note that no "extrathymic sites of T cell development have been identified." (*See*, e.g., *Id.* at page 26, left column at lines 9-10).

Accordingly, at the time of filing of the present application, it was widely appreciated in the art that T-cell development occurred in the thymus and continued into adult life.

Therefore, in light of what was widely known and accepted in the art, at the time of filing of the instant application one of ordinary skill in the art would have accepted that: (1) inflammation is a key mechanism used to isolate, restrict and combat the effects of harmful agents; (2) T cells play a coordinating role in the initiation and maintenance of the inflammatory response; and (3) T cells mature and are selected only in the thymus from birth through adulthood.

Polypeptides of the Invention

In support of the asserted utility of the present invention in the treatment of inflammation, Applicants respectfully direct the Examiner's attention to the teachings of Graf et al., which were previously submitted as Exhibit D on May 9, 2003, in connection with the present application. A second legible copy of Graf et al. is enclosed herewith as Exhibit D for the Examiner's convenience. Graf and coworkers show that polypeptides of the present invention are expressed and play a role in the regulated development of T cells in the thymus.

Graf et al. demonstrate that Tsg expression is upregulated after T-cell receptor activation in developing thymocytes (*See*, Exhibit D, Figure 1), that the components of the BMP signaling pathway are expressed in the thymus (*See*, Exhibit D, Figure 2), and that BMP4 directly inhibits thymocyte development and proliferation (*See*, Exhibit D, Figure 3). The authors then demonstrate that BMP4 inhibition of thymocyte development cannot

be prevented by either Tsg or chordin alone, but that together Tsg and chordin are able to completely reverse this inhibition (*See*, Exhibit D, Figure 5). Graf et al. propose that the coordinated expression of Tsg and chordin in the thymus is required for proper T-cell development and "the balance between BMP2/4, chordin, and Tsg may ensure developmental progression while maintaining a sufficient pool of immature precursors." *See*, Exhibt D at page 170, left column, lines 1-3. Therefore, given the central role of T-cells in immune system function and inflammation, and in view of the teachings of the specification as filed, supported by those of Graf et al. (Exhibit D), Applicants maintain that the present invention is useful as required under 35 U.S.C. § 101.

Basis for the Instant Rejection

The Examiner has alleged that "Applicants have cited the Graf et al. (2202 [*sic*]) publication showing that the Tsg protein modulates BMP2/4 in early Drosophila and vertebrate embryos (see abstract, lines 1-3)." *See*, Paper No. 24 at page 5, lines 12-13. Applicants respectfully suggest that the Examiner may have misinterpreted the statements contained in the abstract of Graf et al. Applicants point out that Graf et al. begin with the following statement:

> The evolutionarily conserved, secreted protein Twisted gastrulation (Tsg) modulates morpho-genetic effects of decapentaplegic (dpp) and its orthologs, the bone morphogenetic proteins 2 and 4 (BMP2/4), in early *Drosophila* and vertebrate embryos.

See, Exhibit D, abstract at lines 1-3. However, Applicants believe that this statement is a summary of previously known facts concerning this molecule, since the authors continue with a description of the data presented in the paper proper:

> We have uncovered a role for Tsg at a much later stage of mammalian development, during T cell differentiation in the thymus ... Tsg can synergize with the BMP inhibitor chordin to block the BMP4-mediated inhibition of

> thymocyte proliferation and differentiation. These data suggest that the developmentally regulated expression of *Tsg* may allow thymocytes to temporarily withdraw from inhibitory BMP signals.

See, Exhibit D, abstract at lines 3-12. Accordingly, Applicants suggest that Graf et al. (Exhibit D) teach the function of this molecule in the development of T-cells in an animal model of the human immune system. These teachings serve to support the assertions of utility as originally made by Applicants on filing of the present application.

The Examiner has characterized the Graf et al. publication as demonstrating that:

> Tsg affects the binding of dpp/BMP2/4 to their cellular receptors and downstream signaling events mediated by the phosphorylation, nuclear translocation, and transcriptional activity of Smad proteins positively or negatively (see page 164, column 1, entire first para) ... study of Tsg is currently limited to early embryonic development (see page 164, column 1, last 5 lines of first para)

See, Paper No. 24 at page 5, line 19 through page 6, line 4. Applicants disagree with the Examiner's analysis of Graf et al., and respectfully point out that the statements identified by the Examiner all occur within the section of the reference known as the introduction, where authors traditionally describe previously known facts concerning the field in which they work. This review of the work of others is identified by the authors in the customary manner of direction to specific reference works identified by numerals contained in parentheses at the conclusion of each sentence of the introduction. A key to the references cited in such a way is found at the conclusion of the reference in a section entitled "References" which begins on page 170, column 1 and concludes on page 171, column 2. One of skill in the art, on reading the teachings of the Graf et al. reference, would appreciate the distinction between what was being summarized by the authors and what was being presented as new data.

Additionally, the Examiner states that "the reference discloses nothing about Tsg being a cytokine, however, the reference discloses that Tsg modulates other cytokines like BMP2/4 (see abstract)." See, Paper No. 24 at page 6, lines 6-7. Applicants respectfully point out that on the date on which the present application was filed, the term "cytokine" was widely accepted to mean a protein secreted by cells to affect the behavior of other cells. (*See*, e.g., Exhibit E, Janeway, C.A. and Travers, P. 'IMMUNOBIOLOGY – The Immune System in Health and Disease', Garland Publishing Inc., N.Y. and London, at page G:5, column 2, lines 44-49 (1994)). Applicants submit that the description of Tsg by Graf et al. meets the definition of cytokine as was accepted in the art as of the filing date of the present application. Accordingly, basis of the present rejection on the supposed discrepancy between the definitions of the claimed polypeptides in the present application and the Graf reference is improper.

The Examiner has further determined that :

> [t]he reference discloses the natural role of Tsg in the thymus (see abstract), however, the instant specification there is not even a hint of such a role for the instant protein ... [a]ccording to the Graf reference, the only time Tsg has an effect is during embryogenesis and in the thymus, however, the instant application fails to even give a hint of what is disclosed in the reference ... the reference discloses the ability of Tsg to act as an antagonist, however, there is no such disclosure of any kind for the polypeptide of the instant invention.

See, Paper No. 24 at page 6, lines 4-14. Applicants respectfully disagree and preliminarily point out that the instant application is not required to support the disclosure of the supporting reference, rather the Graf reference is supportive of the application presently under examination. The present application as originally filed teaches that the claimed polypeptides may be used to treat inflammation, an assertion of utility that is supported by

the state of the art at that date together with the functional characterization presented by Graf et al., and which meets the requirements of 35 U.S.C. § 101.

Applicants point out that the patentability of the present invention, based on this utility, is not dependent on disclosure of the details of how or why the invention works as the Examiner appears to suggest. The Federal Circuit has recently stated with respect to the rejection of claims for lack of utility that:

> "It is not a requirement of patentability that an inventor correctly set forth, or even know, how or why the invention works." Newman v. Quigg, 877 F.2d 1575, 1581, 11 U.S.P.Q.2D (BNA) 1340, 1345 (Fed. Cir. 1989); see also Fromson v. Advance Offset Plate, Inc., 720 F.2d 1565, 1570, 219 U.S.P.Q. (BNA) 1137, 1140 (Fed. Cir. 1983) ("It is axiomatic that an inventor need not comprehend the scientific principles on which the practical effectiveness of his invention rests."). Furthermore, statements that a physiological phenomenon was observed are not inherently suspect simply because the underlying basis for the observation cannot be predicted or explained.

In re Cortright, 49 U.S.P.Q.2d 1464, 1466 (Fed. Cir. 1999). Likewise, according to the axiom of patent law, the utilities asserted for the Human Cytokine Polypeptide do not depend on identification of any receptor or cofactor necessary for its biological activity. Rather, the issue is whether an asserted utility is true. Accordingly, the basis of the present rejection, on the supposed lack of disclosure of a molecular mechanism by which the claimed polypeptides may be used to treat inflammation, is improper.

The Examiner appears to have further based the present rejection on the fact that "the Graf reference was published in 2002, six years after the earliest filing date of the instant application. Applicants are reminded that an invention must be complete as filed." *See*, Paper No. 24 at page 5, lines 14-18.

Applicants respectfully traverse this basis for the present rejection, and assert that scientific papers may be used to corroborate Applicants' asserted utility. Legal precedent

for the use of post-filing date references in this manner can be found in *In re Brana*, where the courts stated:

> The Kluge declaration, though dated after applicants' filing date, can be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification. *In re Marzocchi,* 439 F.2d at 224 n.4, 169 U.S.P.Q. (BNA) at 370 n.4.

See, *In re Brana*, 51 F.3d 1560 at 1567 n.19, 34 U.S.P.Q.2D (BNA) 1436 (March 30, 1995). Accordingly, Applicants submit that the asserted utilities are supported by an independent third party publication, that of Graf et al. which is attached hereto as Exhibit D. As discussed in detail above, Graf et al. show that polypeptides of the present invention are expressed and play a role in the regulated development of T cells in the thymus. Accordingly, Graf et al. provide independent support for Applicants' assertion that the polypeptides of the invention may be used to treat inflammation.

The Examiner maintains rejection of the pending claims under 35 U.S.C. § 101, as allegedly "Applicants disclose in the specification that the claimed protein has homology to the 'Tsg' protein ... knowledge of the overall structure or domain family is still not enough to confidently assign function to the protein." *See*, Paper No. 24, page 4.

Applicants respectfully disagree and traverse. Even assuming, *arguendo*, that the Examiner is correct in this assertion and that homology is not predictive of function, the ability to predict function based on sequence homology has no bearing on the current set of circumstances. In the present case an assertion has been made that polypeptide of the invention may be used to treat inflammation. As described in detail above, inflammation is a specific process regulated and maintained by T-cells, which develop at a single site and by a single pathway that is regulated by polypeptides of the instant invention. (*See*, Exhibits A-D). Accordingly, regardless of whether protein function may be predicted

correctly or not, the assertion that the claimed polypeptides may be used to treat inflammation is more likely than not true.

The Examiner further alleges that "the specification fails to provide any further disclosure about the use of the polypeptide in the treatment ... [t]he possibility of the employment of a protein of the instant invention to treat inflammation is not a substantial or specific utility." *See*, Paper No. 24 at page 5.

Once more Applicants respectfully disagree and traverse. Applicants contend that determination of the ability of any polypeptide, including the polypeptides of the instant invention, to regulate the inflammatory response was routine to one of skill in the art as of the date on which the present application was filed. In support of this contention, Applicants respectfully direct the Examiner's attention to the teachings of attached Exhibits A-C together with references identified therein. Upon appreciation of the ability of the claimed polypeptides to regulate the inflammatory response, it would have required only routine procedures for one of skill in the art to use the claimed compounds for their intended use. Furthermore, demonstration of clinical efficacy in the treatment of inflammation is not a requirement for patentability. Knowledge of a biological or pharmacological activity of a compound is beneficial to the public, and "adequate proof of any such activity constitutes a showing of practical utility." *Nelson v. Bowler*, 626 F.2d 853, 856 (C.C.P.A. 1980). Applicants disclose in the specification the credible assertion that the Human Cytokine Polypeptide of the present invention may be useful to treat inflammation. Moreover, Applicants have provided evidence that the Human Cytokine Polypeptide of SEQ ID NO:2 regulates T-cell development, a critical aspect of inflammation. As such, Applicants submit that adequate proof of a biological activity of the Human Cytokine Polypeptide has been shown, thereby constituting a showing of practical utility.

In view of the above, the assertions that Human Cytokine Polypeptide would act to regulate immune cell development and homeostasis, and would be useful as a means of treatment in inflammation, would *not* be incredible to one skilled in the art. That is, since the facts upon which the assertions are based are consistent with the logic underlying the assertions, Applicants submit that one of ordinary skill in the art would *not* reasonably doubt Applicants' assertions regarding utility.

The Examiner has further alleged that:

> the specification describes only vague and hypothetical uses for the claimed protein. The present specification only presents an invitation to further characterize the claimed invention and to identify practical uses for the protein providing only vague guidance as to where or how such efforts should be directed."

See, Paper No. 24 at page 6, lines 17-20. Applicants respectfully disagree and traverse such characterization of the present disclosure.

As described in detail above, the present application teaches that the claimed polypeptides may be used to treat inflammation. Applicants fail to see how this asserted use can be interpreted as being either "vague" or "hypothetical." Rather, this asserted use is clear and definite in the sense that treating inflammation may be readily defined and distinguished from utilities such as treating conditions other than inflammation.

Furthermore, the assertion of utility is not "an invitation to further clarify the claimed invention," but rather it is a definite statement of the usefulness of the present invention. Applicants respectfully point out that utility can exist for therapeutic inventions "despite the fact that an applicant is at a very early stage in the development of a pharmaceutical product or therapeutic regimen based on a claimed pharmacological or

bioactive compound or composition." M.P.E.P. § 2107 (III) at 2100-27. "Usefulness in patent law . . . necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans." *In re Brana*, 51 F.3d 1560, 1568 (Fed. Cir. 1995).

Further, Applicants do not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty or provide actual evidence of success in treating humans where such a utility is asserted. *See* M.P.E.P. § 2107.02 (I) at 2100-34. All that is required of Applicants is that there be a *reasonable* correlation between the biological activity and the asserted utility. *See Nelson v. Bowler*, 626 F.2d at 857.

Based upon the general knowledge of those skilled in the art that the regulation of immune cell homeostasis is directly implicated in the development of inflammation, it would be reasonable to expect that Human Cytokine Polypeptide of the present invention could be utilized in the treatment of inflammation. As such, based on the totality of the evidence, an artisan of ordinary skill in the art of molecular biology would find the statements of utility contained in the specification to be credible.

The Examiner further states that it:

> cannot be presumed that a chemical compound is "useful" under § 101 ... simply because the compound is closely related only in a structural sense to other compounds known to be useful. Also, that nebulous descriptions of biological properties or activities of a compound in a specification do not convey an explicit indication of the usefulness of the compound
>
> ...
>
> it is not enough that the specification disclose that an intermediate can be used to produce some intended product of no known use. Nor is it enough that the product disclosed to be obtained from the intermediate belongs to some class of compounds which now is, or in the future might be, the subject of research to determine some specific use."

See, Paper No. 24 at page 7, lines 1-9. Applicants respectfully disagree and traverse this basis for rejecting the instant claims.

As described in great detail above, the utility of the presently claimed polypeptides does not rely on a close relatedness "only in a structural sense to other compounds known to be useful," nor does it rely on "nebulous descriptions of biological properties or activities" of said polypeptides. Rather, the specification contains Applicants' explicit assertion that the compounds of the invention may regulate immune cell development and homeostasis, and would be useful as a means of treatment in inflammation. Furthermore, the instant claims are not directed to a product or any intermediate that "can be used to produce some intended product of no known use," or that "belongs to some class of compounds which now is, or in the future might be, the subject of research to determine some specific use." Rather the claims encompass polypeptides which themselves may regulate immune cell development and homeostasis and be used in the treatment of inflammation. Accordingly, Applicants contend that the utterances of the courts relied upon by the Examiner in making the present rejections are not relevant to the instant set of circumstances and are therefore being misapplied out of their intended contexts.

Even assuming, *arguendo*, the Examiner has established a *prima facie* showing that the claimed invention lacks utility, Applicants respectfully submit that they have rebutted the Examiner's showing by proffering sufficient evidence to lead one skilled in the art to conclude that the asserted utilities are more likely than not true. Applicants have supplied evidence that the Human Cytokine Polypeptide of SEQ ID NO:2 is identical to a molecule, *i.e.*, Tsg, that acts as a regulator of T-cell development and homeostasis and is likely involved in inflammation.

In view of the facts set out above, Applicants assert that a skilled artisan would not reasonably doubt that polypeptides comprising the Human Cytokine Polypeptide amino acid sequence shown in SEQ ID NO:2 can be used in the treatment of inflammation. As such, Applicants assert that the presently claimed invention possesses a credible utility that constitutes a patentable utility under 35 U.S.C. § 101.

In view of the above, Applicants submit that the asserted utilities of the invention meet the statutory requirement set forth in 35 U.S.C. § 101. The Examiner has failed to establish and maintain grounds as to why a rejection for lack of utility is proper. Accordingly, Applicants respectfully request that the rejection be withdrawn.

The Examiner has also rejected claims 25-79 under 35 U.S.C. § 112, first paragraph, "since the claimed invention is not supported by either a substantially asserted utility or a well-established utility for the reasons set forth above, one skilled in the art clearly would not know how to use the claimed invention". *See*, Paper No. 24 at page 7.

Applicants respectfully disagree and traverse this rejection.

As detailed above, the asserted utilities of the invention meet the statutory requirement set forth in 35 U.S.C. § 101 and, armed with the specification of the instant invention, one skilled in the art clearly would know how to use the claimed invention. Accordingly, Applicants respectfully request that the rejection of claims 25-79 under 35 U.S.C. § 112, first paragraph, be withdrawn.

CONCLUSION

Applicants respectfully request consideration and entry of the foregoing remarks into the file. Applicants believe that no fee is due in connection herewith; however, should the Patent Office determine otherwise, please charge the required fee to Human Genome Sciences, Inc., Deposit Account No. 08-3425.

Respectfully submitted,

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Enclosures
JMM/BM

Twisted gastrulation is a conserved extracellular BMP antagonist

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Bone morphogenetic protein (BMP) signalling regulates embryonic dorsal–ventral cell fate decisions in flies, frogs and fish[1]. BMP activity is controlled by several secreted factors including the antagonists chordin and short gastrulation (SOG)[2,3]. Here we show that a second secreted protein, Twisted gastrulation (Tsg)[4], enhances the antagonistic activity of Sog/chordin. In *Drosophila*, visualization of BMP signalling using anti-phospho-Smad staining[5] shows that the *tsg* and *sog* loss-of-function phenotypes are very similar. In S2 cells and imaginal discs, TSG and SOG together make a more effective inhibitor of BMP signalling than either of them alone. Blocking Tsg function in zebrafish with morpholino oligonucleotides causes ventralization similar to that produced by chordin mutants. Co-injection of sub-inhibitory levels of morpholines directed against both Tsg and chordin synergistically enhances the penetrance of the ventralized phenotype. We show that Tsgs from different species are functionally equivalent, and conclude that Tsg is a conserved protein that functions with SOG/chordin to antagonize BMP signalling.

TSG is required to specify the dorsal-most structures in the *Drosophila* embryo, for example amnioserosa[4]. Mutations in the BMP-like ligands, Decapentaplegic (DPP) and Screw (SCW), the BMP inhibitory factor SOG, or the SOG-processing enzyme Tolloid (TLD), also cause loss of the amnioserosa, even though some of these products seem to have opposing biochemical functions[2,6–8]. To place TSG activity relative to the biochemical function of these other factors, we examined its loss-of-function phenotype using molecular markers (Fig. 1A). The phenotype of *tsg* mutants (Fig. 1A, c, g, l) is most similar to that produced by loss of the BMP antagonist SOG (Fig. 1A, b, f, k) rather than that produced by loss of the ligands DPP or SCW (data not shown), or the SOG-processing protease TLD (Fig. 1A, d, h, m). In both *tsg* and *sog* mutants, the dorsal marker Rhomboid (*rho*) expands (Fig. 1A, b, c)[9], whereas in *tld* mutants no *rho* expression is observed (Fig. 1A, d)[8]. In contrast, mutations in *tsg*, *sog* and *tld* eliminate expression of other presumptive amnioserosa markers including Race (Fig. 1A, g, f, h), Hindsight (data not shown) and Zerknüllt (*zen*) (data not shown). To determine whether the response of these is indicative of different threshold levels of DPP signalling[10], we used an anti-phospho-Smad antibody[5] to directly visualize the levels of ligand signalling. Wild-type embryos accumulate phosphorylated mother against DPP (P-MAD) in an 18–20-cell-wide dorsal stripe at mid-cellulariza-

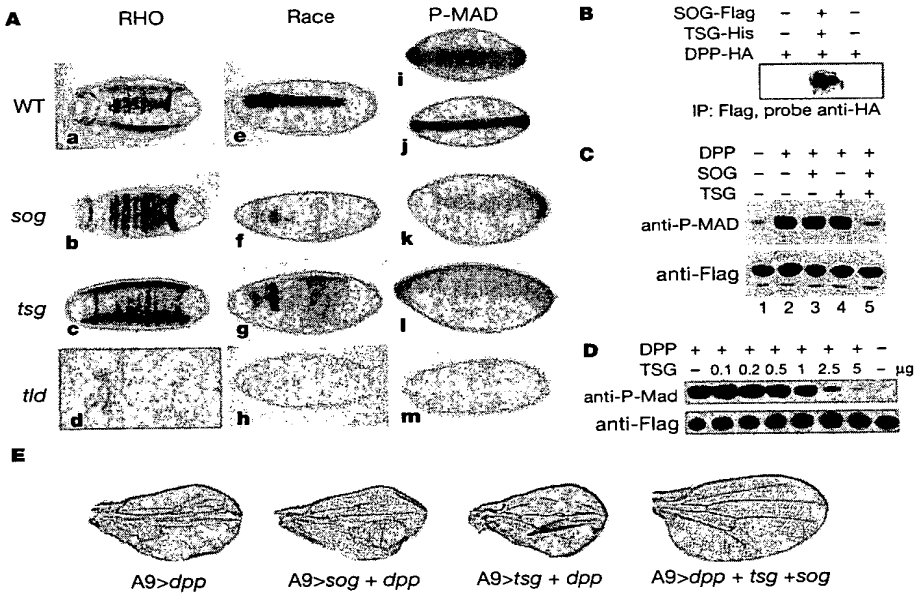

Figure 1 Tsg and SOG synergistically inhibit DPP signalling. **A**, The *tsg* loss-of-function phenotype is similar to that of *sog*. **a–h**, *In situ* hybridization of a Rhomboid RNA probe and a Race RNA probe to wild-type (WT), sog^{YSO6}, tsg^{XB86} and tld^{B4} mutant embryos. Mutant embryos were identified by the absence of *lacZ* expression supplied by a marked balancer chromosome. All embryos are at the mid-cellular-blastoderm or early gastrulation stage; anterior is to the left and the view is dorsal. **i–m**, Anti-phospho-MAD staining of WT (**i**, **j**), sog^{YSO6} (**k**), tsg^{XB86} (**l**) and tld^{B4} (**m**) mutant embryos. The embryos in **i** and **j** are dorsal side up, and in **k–m** they are viewed laterally. **B**, TSG and SOG form a high-affinity complex with DPP. S2 cells were transfected with DPP-HA and either SOG alone or SOG and TSG. After induction with Cu^{+2}, the supernatants were harvested and immunoprecipitated (IP) with anti-Flag antibody, transferred to a PVDF membrane and probed with anti-HA (12CA5 Roche) antibody as described[9]. **C**, TSG and SOG synergistically inhibit DPP signalling in S2 cells. In lanes 2–5, 20 ng purified DPP was added. Lane 1 was mock treated with buffer. In lanes 3–5, 1.25 µg SOG, 1 µg TSG or 1 µg of each were premixed with DPP and added to cells. The top panel was probed with anti-P-MAD antibody; the bottom with anti-Flag antibody. **D**, TSG alone inhibits DPP signalling in S2 cells at high concentrations. Cells were transfected with MAD-Flag as above, and then treated with 20 ng DPP and the indicated levels of TSG. **E**, TSG and SOG together form an effective inhibitor of DPP *in vivo*. Transformant lines containing UAS *dpp*, *sog* and *tsg* constructs[9,30] were crossed in the combinations indicated to the GAL4-A9 driver[30].

tion (Fig. 1A, i) that rapidly resolves into an 8–9-cell-wide stripe (Fig. 1A, j) of more intensely stained cells just as gastrulation starts. Although an underlying gradient of activity not detectable by this method may exist[7,11,12] these results instead suggest that DPP/SCW activity is distributed in a sharp on–off pattern that resolves into a narrow stripe of dorsal cells, which—posterior to the cephalic furrow—corresponds in width to those cells labelled by the amnioserosa markers Race and Hindsight. In *sog* and *tsg* mutants (Fig. 1A, k, l), P-MAD fails to refine and intensify, whereas in *tld* mutants (Fig. 1A, m) P-MAD activity is below the level of detection in all dorsal cells. We suggest that the low, uniform levels of P-MAD seen in *sog* and *tsg* mutants are sufficient to activate *rho*, but not *race*, *hnd* or *zen* transcription.

As the phenotypes of *tsg* and *sog* mutants are similar, we sought to determine whether TSG can enhance the binding of SOG to ligand. Co-immunoprecipitation of DPP by SOG is greatly enhanced when these two factors are coexpressed in S2 cells along with TSG (Fig. 1B). To test whether the combination of SOG and TSG blocks DPP signalling better than SOG alone, we developed an S2 cell-culture assay for DPP signalling (Fig. 1C). At high concentration TSG alone can block DPP signalling (Fig. 1D); however, at lower concentration, the combination of TSG and SOG together dramatically reduces the DPP-dependent accumulation of P-MAD much more efficiently than either could alone. *In vivo* overexpression of *sog* and *tsg* together can completely reverse the phenotype of ectopic *dpp* expression in the wing, whereas the expression of either alone has no effect. We conclude that a complex of TSG and SOG is an efficient antagonist of DPP signalling.

To determine whether Tsg is conserved among other species, we sought and found genes in the database related to *Drosophila* TSG in human, mouse, zebrafish and *Xenopus*. In addition, we found a second *tsg*-related sequence in *Drosophila* (*tsg2*) and obtained a second zebrafish *tsg* (*tsg1*) using degenerate polymerase chain reaction (PCR) methods. The protein products show extensive similarity with about 50% of 202 amino-acid residues matching in all four species (see http://darwin.bio.uci.edu/~marshlab/). The pairs of *tsg* genes in fly and fish are closer to each other than to *tsg* in any other species, suggesting independent gene-duplication events in these two species. We mapped the human, mouse and zebrafish (*tsg1*) genes by a combination of fluorescence *in situ* hybridization (FISH) or radiation hybrid mapping. The mouse gene maps to 17E1.3–E2, a region that is syntenic to 18p11.2–3 where the human homologue resides. In zebrafish, *tsg1* is located at linkage group 24-74.5, which is syntenic to the human locus and indicates that all

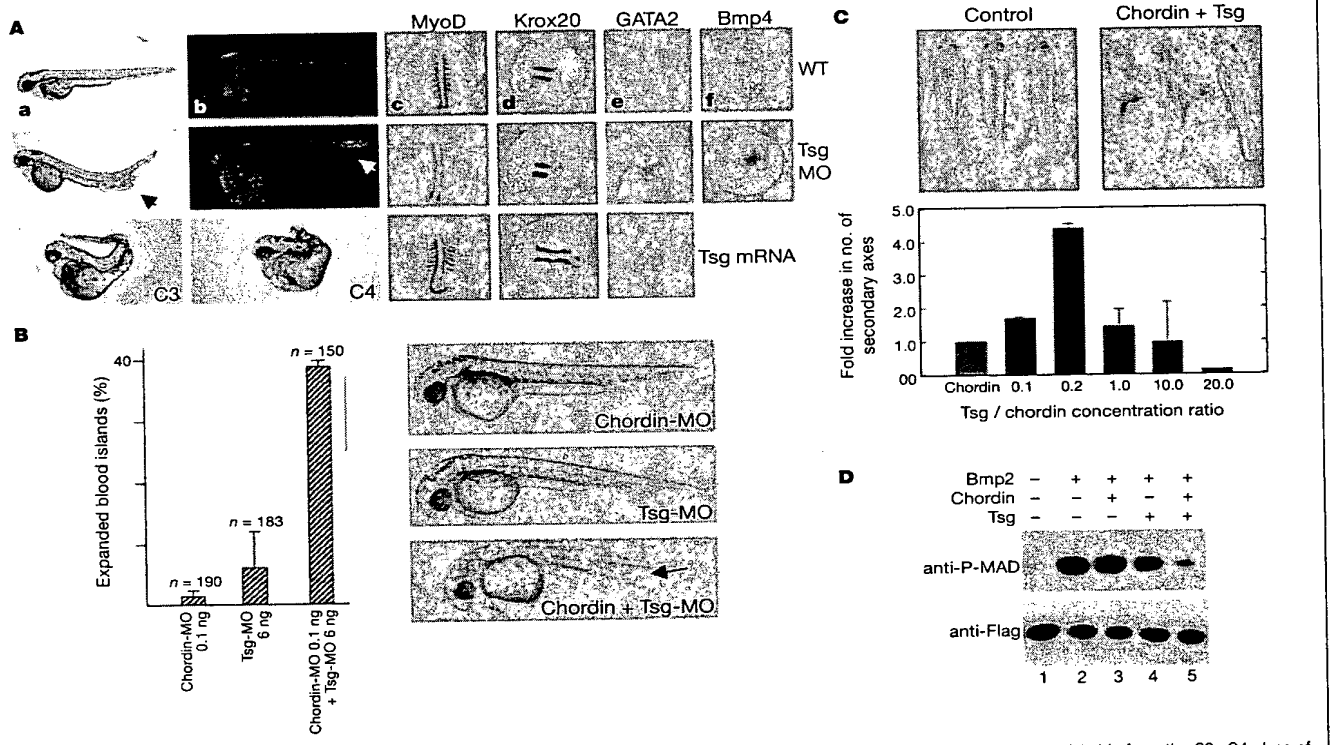

Figure 2 Vertebrate Tsg enhances chordin's antagonist function. **A**, Loss of zebrafish *tsg1* activity ventralizes zebrafish embryos whereas ectopic *tsg1* mRNA has dorsalizing activity. **a**, WT embryo (48 h). **b**, WT embryo injected with 9 ng UroD morpholino oligonucleotides. Blood cells are indicated by red staining. **c–f**, *In situ* hybridization. *myoD* (8 somite stage) (**c**); *krox20* (8 somite stage) (**d**); *GATA2* (22 somite stage) (**e**); and *bmp4* (tail bud view, 3 somite stage) (**f**). Second row as above but injected with 12 ng *tsg1* morpholino oligonucleotide. The filled and open arrows indicate ectopic blood islands (60%, $n = 110$, filled arrow; 60%, $n = 100$, open arrow). Caudal expression of *bmp4* is expanded at the 3 somite stage (48%; $n = 25$), and the blood marker *GATA2* is expanded at the 22 somite stage of development (48%; $n = 21$). Paraxial expression of MyoD is significantly reduced at the 8 somite stage (38%; $n = 33$), and the anterior ectodermal marker Krox20 is moderately reduced at the 8 somite stage (49%; $n = 39$). The third row shows embryos after injection with zebrafish Tsg1 mRNA. At 48 h of development, Tsg1 mRNA-injected embryos display phenotypes indistinguishable from the C3–C4 class of dorsalized mutants[17]. Expression of *myoD* (50%; $n = 8$) and *krox20* (70%; $n = 10$) is also significantly expanded at the 8 somite stage, while *GATA2* and *bmp4* are reduced. **B**, Enhancement of the zebrafish Tsg1 loss-of-function phenotype by sub-inhibitory loss of the chordin gene. Embryos were injected with a low dose of zebrafish Tsg1-MO, chordin-MO, or both, and assayed for blood island expansion (arrow). **C**, Effect of *Xenopus Tsg* on the ability of chordin to block BMP signalling in *Xenopus*. Chordin and Tsg were injected at the ratios indicated. The graph represents the combined results from five experiments. About 200 embryos were injected for each point. On the *y* axis 1.0 for chordin corresponds to 2–16% induction of secondary axes. The chordin mRNA concentration was 5 pg. **D**, Vertebrate Tsg and SOG synergistically inhibit BMP-2 signalling in *Drosophila* S2 cells. The experiment was carried out as in Fig. 1. The mouse protein concentrations were: *bmp2*, 0.2 ng; chordin, 1 μg; and Tsg, 0.5 μg.

three genes are probably functional orthologues.

The zebrafish *tsg1* gene is expressed uniformly in early embryos, whereas zebrafish *tsg2* is only expressed at later stages (data not shown). Hence, we focused our analysis on zebrafish *tsg1* and used morpholino oligonucleotides[13] to reduce the function of this gene in early zebrafish development. Injection of a *tsg1* morpholino oligonucleotide (*ztsg*-MO) produces a phenotype characteristic of expanded BMP signalling (Fig. 2A)[14,15]. Using morphological criteria and fluorescent red blood cells[13], we found that embryos develop expansions of the ventral fin region that correspond to ectopic blood islands (Fig. 2A, arrowheads), a tissue derived from ventral mesoderm. Injected embryos also show an expansion of *GATA2*, loss of paraxial mesoderm (visualized with the marker *myoD*), and a mild reduction of anterior ectodermal tissues (detected by staining for *krox20*). Caudal expression of *bmp4* is also expanded in these embryos (Fig. 2A), while the anterior ectodermal marker *otx2* is reduced (data not shown). Treated embryos also exhibit an expansion in apoptotic cells ventral to the yolk extension (data not shown), similar to *dino* and *mercedes* mutants[14,16]. Overall, this phenotype is very similar to that of *ogon/mercedes* mutants[14,15] and moderate *chordin* loss-of-function mutants, and represents a modest ventralized phenotype[13,14].

Increasing the level of zebrafish *tsg1* by injecting messenger RNA produced phenotypes characteristic of diminished BMP signalling[17–19] including reduced axial length with loss of ventral fin (Fig. 2A), an expansion of *myoD* and *krox20*, and a reduction in *GATA2*. This is a phenocopy of the C3–C4 class of dorsalized mutant embryos, similar to that of the Snailhouse (BMP7 homologue) and Piggytail mutations[17,19]. Furthermore, the dorsalizing effect of zebrafish Tsg1 mRNA partially reverses the ventralizing effect of *tsg1* (9 ng *tsg1*-MO caused 47 ± 2%, n = 376, ventralized embryos; 9 ng tsg1-MO plus 30 pg Tsg1 mRNA resulted in 19 ± 8%, n = 270, ventralized embryos) suggesting that loss of *tsg1* is responsible for the phenotype. We conclude that loss of *tsg1* leads to embryos with a ventralized phenotype, whereas ectopic expression of *tsg1* leads to a dorsalized embryonic phenotype.

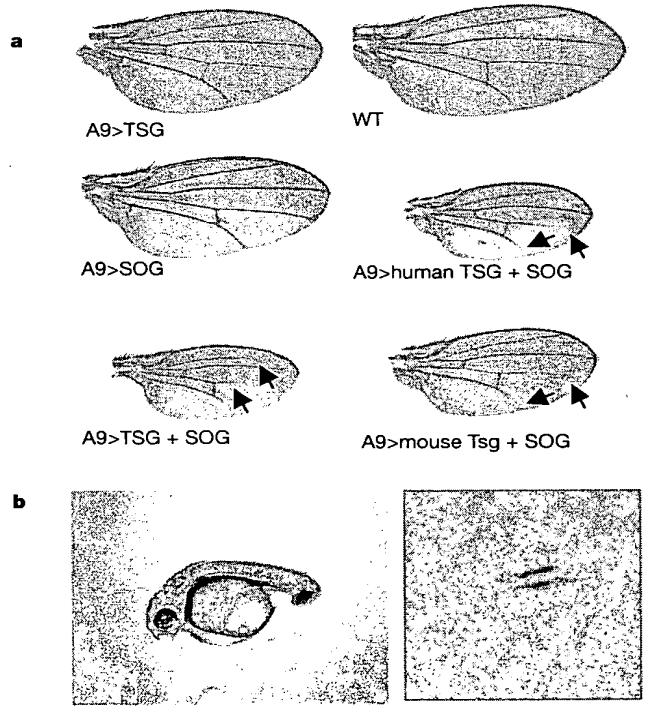

Figure 3 Functional equivalence of Tsg proteins. **a**, The GAL4-A9 driver was crossed to flies at 25 °C (first column) or 29 °C (second column) with the indicated transgenes. Arrows indicate loss of distal vein material. **b**, Injection of 30 pg *Drosophila* Tsg into zebrafish embryos results in a C3-like dorsalized phenotype (54%; n = 22) at 48 h after fertilization (left). Right, embryo injected with 30 pg *Drosophila tsg* exhibiting expansion of *krox20* at the 8 somite stage (compare with Fig. 2a).

As our *Drosophila* data suggested that one function of TSG is to co-operate with SOG to inhibit BMP signalling, we asked whether the same relationship is true in vertebrates by determining whether a modest reduction of zebrafish *chordin* activity could enhance the effect of a moderate reduction in *tsg1* activity. Sub-inhibitory levels of a zebrafish *chordin* morpholino oligonucleotide and *tsg1*-MO were injected into wild-type embryos, and the effect on ectopic blood island development was scored. These two morpholino oligonucleotides synergistically enhanced blood island expansion (Fig. 2B), supporting the view that both of these gene products co-operatively inhibit BMP signalling. As with the *Drosophila* components, we found that the combination of purified mouse chordin and Tsg was better able to inhibit mouse BMP-stimulated phosphorylation of Mad in S2 cells than either could alone (Fig. 2D).

We also tested for synergy between Tsg and chordin mRNA in *Xenopus* embryos by co-injecting their mRNAs and scoring for enhancement of secondary axis formation[20]. Co-injection of *Xenopus* Tsg and chordin reveals a dose–response optimum. When a sub-inhibitory dose of chordin mRNA is supplemented with increasing levels of Tsg mRNA, the fraction of embryos exhibiting a secondary axis increases up to 4.5-fold over chordin alone at a 1/5 ratio of Tsg/chordin mRNA. However, if the Tsg/chordin ratio is increased to 1:1 or higher, the number of secondary axes is reduced to basal levels and the resulting tadpoles have normal morphology. Injection of 150 pg Tsg alone (the highest concentration of Tsg mRNA used in these experiments) had no effect on embryonic development. Notably, if we increase the level of Tsg relative to chordin in the S2 experiments, we do not see a reversal of the inhibition phenotype (data not shown), suggesting that additional factors probably modulate the *in vivo* response. Taken together, we conclude that, like *Drosophila* TSG, vertebrate Tsg can co-operate with chordin to inhibit BMP signalling.

As a final test of the functional equivalence of the vertebrate and invertebrate *tsg* genes, we expressed the human and mouse genes under the control of the UAS promoter in flies, and injected *Drosophila* TSG mRNA into zebrafish embryos. The phenotype of animals expressing human *TSG* and *Drosophila sog* in wing discs (Fig. 3a) resembles that of *dpp* shortvein alleles[21] and is very similar to that produced by coexpression of the *Drosophila tsg* and *sog* genes (Fig. 3a; see also ref. 9). When injected into zebrafish, *Drosophila tsg* produces a dorsalized phenotype equivalent to that produced by zebrafish *tsg1*, which includes reduced axial length and expansion of *krox20* (Fig. 2A; compare with Fig. 3b) and *myoD* (data not shown).

Our experiments, and those of others[22–24], suggest that Tsg has three molecular functions. First, it can synergistically inhibit Dpp/BMP action in both *Drosophila* and vertebrates by forming a tripartite complex between itself, SOG/chordin and a BMP ligand (Fig. 1B, see also refs 9, 24). Second, Tsg seems to enhance the Tld/BMP-1-mediated cleavage rate of SOG/chordin and may change the preference of site utilization (O.S. and M.B.O., unpublished observations; see also refs 9, 23). Third, Tsg can promote the dissociation of chordin cysteine-rich (CR)-containing fragments from the ligand[24]. Different organisms may exploit each of these properties to different degrees during development depending on the relative *in vivo* concentrations of each molecule. We propose that in *Drosophila* and zebrafish the primary function of Tsg is to form a tripartite complex between itself, Sog/chordin and a BMP ligand. In *Drosophila*, this complex acts to redistribute a limiting amount of DPP, such that activity is elevated dorsally at the expense of being lowered laterally. The net driving force for this redistribution is likely to be diffusion of SOG from its ventral source of synthesis[25].

This is consistent with the finding that SOG diffusion is essential for activation of genes such as *race* that require high levels of DPP/SCW signalling (Fig. 1, see also ref. 26). In this model TLD would serve to modulate both the net movement of DPP and its release from the inhibitory complex by cleaving SOG[8,25]. The ability of TSG to enhance the rate of SOG cleavage may also be an important aspect of this model in that it helps ensure the proper timing of these rapid developmental events. It seems unlikely that TSG is needed to remove an inhibitory CR-containing fragment from DPP as the affinity of full-length SOG for DPP in the absence of TSG seems to be low. Likewise, in zebrafish the phenotype of reduced Tsg function is ventralized and not dorsalized as would be predicted if Tsg were primarily needed to release inhibitory CR fragments from ligand. In *Xenopus*, however, perhaps the endogenous levels of full-length chordin and CR fragments are higher than in zebrafish, thereby making the CR displacement activity of Tsg the more important biological function[24]. Determination of the *in vivo* levels of these proteins, along with a more careful analysis of the concentration optima for each type of reaction involving Tsg function, will be required before we can fully understand all of its *in vivo* activities. □

Methods

Isolation of *tsg* clones and gene mapping

Human *TSG* complementary DNA clones (accession numbers AW160804, AA905905, AI222228, AA486291, AI018381, AI379897 and AA758784) were obtained from Research Genetics. One clone (AI018381) was sequenced in its entirety, additional 5′ sequence was obtained from published Est sequences. Mouse *Tsg* cDNA clone (accession number AW258143) was also obtained from Research Genetics. The zebrafish *tsg1* was isolated from an epiboly cDNA library (S. Ekker) using two degenerate primers (5′ primer: 5′-TG(CT)TG(CT)AA(AG)GA(CTAG)TG(CT)(CTA)T-3′ and 3′ primer: 5′-CC(CTG)A(CT)(AG)GA(CT)TC(AG)CA(AG)CA-3′). These primers amplified a 0.5-kilobase (kb) fragement that was used as a probe to identify a 1.2 kb cDNA from a zebrafish epiboly library. We sequenced this clone using standard methods. The human *TSG* locus was mapped against the Stanford G3 hamster–human radiation hybrid panel using primer pairs at the beginning, middle and end of the *TSG* mRNA. This placed human *TSG* between STS markers D18, and D18 within cytogenetic band 18p11.2. The mouse *Tsg* was mapped by FISH using a 16-kb genomic mouse *Tsg* fragment as a probe. The chromosomal assignment and band designation were determined by sequential G-banding to FISH. The zebrafish (*Danio rerio*) *tsg1* gene was mapped to linkage group 24 at 74.5 cM using a mouse–fish radiation hybrid panel[27].

Altering signal peptides

While conducting these studies, we found that the secretion signals of the mammalian and *Drosophila* genes are incompatible with the other species. To circumvent these secretion-related problems, we used PCR to replace the human and mouse signal sequences with the *Drosophila* sequence and also the *Drosophila* signal peptide with the zebrafish sequence (details are available on request).

Production and purification of recombinant proteins

Recombinant proteins SOG-Myc, Tsg-His and Dpp-haemagglutinin (HA) were produced as described[9]. Conditioned medium containing SOG-Myc was applied to a 1 × 10-cm^2 S-Sepharose column (Pharmacia) equilibrated with 100 mM MOPS-Na, pH 6.0 (buffer A). After washing with buffer A containing 300 mM NaCl, the column was eluted with buffer A containing 750 mM NaCl, and the fractions were combined and stored for further use. Conditioned medium containing Tsg-His was applied to a 1 × 10-cm Q-Sepharose column (Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.5 (buffer B). After washing with buffer B containing 200 mM NaCl, the column was eluted with buffer B containing 500 mM NaCl, and the fractions were combined and applied to a 1 × 4-cm Ni-NTA agarose column (QIAGEN) equilibrated with 100 mM Tris-HCl, pH 8.0 (buffer C). After washing with buffer C containing 1 M NaCl, the column was eluted with buffer C containing 100 mM imidazole. Fractions containing Tsg-His were pooled and dialysed against 50 mM Tris-HCl, 150 mM NaCl, pH 7.4.

Signalling assays

Ten micrograms of Flag-tagged MAD were transfected in S2 cells at 2×10^7 cells per dish. After 3 days, the cells were collected and split into 20 samples. One microgram Tsg-His and/or 1.25 μg SOG-Myc (Fig. 1C), or 0.5 μg mouse Tsg-protein C and/or 1 μg chordin-His (R&D Systems) (Fig. 2D) were premixed for 3 h at room temperature (RT) with 10^{-9} M Dpp or 10^{-11} M BMP2 (R&D Systems) and then incubated with S2 cells expressing Flag-Mad for 3 h at RT. The cells were spun down and lysed by 1 × SDS–PAGE buffer. The supernatants were separated by SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membrane was probed with anti-Phospho Mad PS1 antibody at 1/5,000 dilution[5] and anti-Flag M2 antibody (Kodak) at 1/2,000 dilution, followed by incubation in secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse; Jackson Laboratory) and developed using ECL substrate (Pierce).

Morpholino oligonucleotides

We obtained Morpholino oligonucleotides from Gene Tools, LLC (Corvallis). We selected sequences on the basis of the design parameters recommended by the company. The zebrafish chordin morpholino oligonucleotide was as described[13]. *tsg1*-MO 5′-CTGATG ATGATGATGAAGACCCCAT-3′.

Embryo manipulations and microinjections

Morpholino oligonucleotides were injected as described[13]. For *Xenopus* injections, embryos were obtained by *in vitro* fertilization and cultured as described[28]. Microinjections of mRNA were performed at the 4-cell stage in 0.3 × MMR, 3.5% Ficoll. We determined dorsal–ventral polarity of early cleavage stage embryos using pigmentation differences[28].

In situ hybridization and antibody staining

Hybridization to *Drosophila* and zebrafish embryos was as described[4,29]. The rabbit anti-phospho Mad antibody was a gift from P. ten Dijke and used at 1/2,000 dilution. Staining was visualized using an alkaline phosphatase-coupled secondary antibody (Promega Laboratories).

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Twisted gastrulation can function as a BMP antagonist

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Bone morphogenetic proteins (BMPs), including the fly homologue Decapentaplegic (DPP), are important regulators of early vertebrate and invertebrate dorsal–ventral development[1–6]. An evolutionarily conserved BMP regulatory mechanism operates from fly to fish, frog and mouse to control the dorsal–ventral axis determination. Several secreted factors, including the BMP antagonist chordin/Short gastrulation (SOG)[7–12], modulate the activity of BMPs. In *Drosophila*, Twisted gastrulation (TSG) is also involved in dorsal–ventral patterning[13–15], yet the mechanism of its function is unclear. Here we report the characterization of the vertebrate Tsg homologues. We show that Tsg can block BMP function in *Xenopus* embryonic explants and inhibits several ventral markers in whole-frog embryos. Tsg binds directly to BMPs and forms a ternary complex with chordin and BMPs. Coexpression of Tsg with chordin leads to a more efficient inhibition of the BMP activity in ectodermal explants. Unlike other known BMP antagonists, however, Tsg also reduces several anterior markers at late developmental stages. Our data suggest that Tsg can function as a BMP inhibitor in *Xenopus*; furthermore, Tsg may have additional functions during frog embryogenesis.

We isolated human Twisted gastrulation (TSG) in a screen for secreted factors, and mouse and *Xenopus* Tsg by low-stringency hybridization using human TSG as the probe. These vertebrate Tsgs have a high sequence homology to each other (more than 80% identical) and are about 30% identical to *Drosophila* TSG at the amino-acid level (data not shown). Tsg is expressed maternally and in all developmental stages in *Xenopus*, and at least from gastrula stages onward in mouse (data not shown). Expression of Tsg is also detected in a variety of adult tissues in both mouse and human (data not shown).

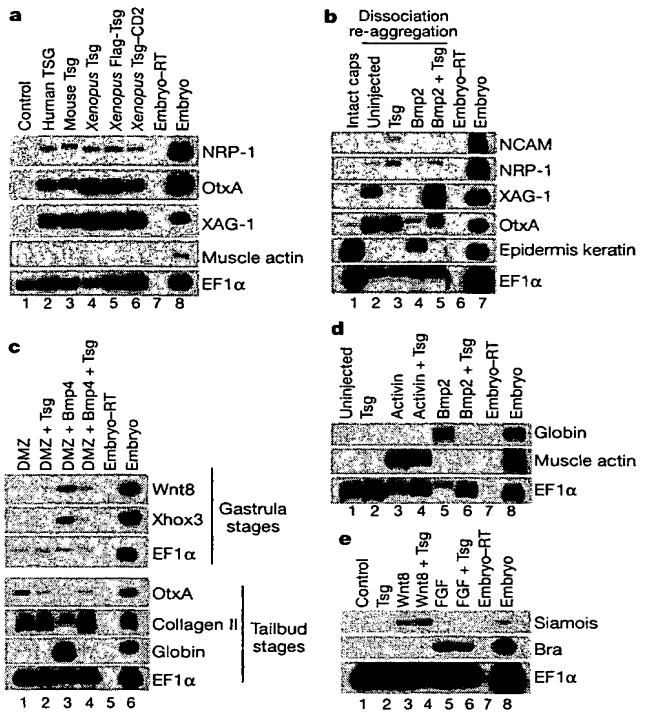

Figure 1 Vertebrate Tsgs inhibit BMP signalling in embryonic explants. **a**, Vertebrate Tsgs induce expression of cement gland and neural markers in animal caps. **b**, *Xenopus* Tsg blocks epidermal induction and neural inhibition by Bmp2 in dissociated animal-cap cells. **c**, *Xenopus* Tsg blocks ventralization of dorsal marginal-zone explants by Bmp4. **d**, *Xenopus* Tsg blocks mesodermal induction by Bmp2, but not activin. **e**, *Xenopus* Tsg does not interfere with the Wnt or FGF signalling. In animal-cap assays, RNAs were injected into animal poles of both cells of 2-cell-stage embryos. Animal caps were dissected at blastula stages (stage 9) and incubated to gastrula (stage 11, **e**) or neurula stages (stage 20, **a**, **b**, **d**). In **b**, the caps were dissociated at blastula stages for 4 h before re-aggregation and incubation to neurula stages, as described[16]. In the marginal-zone assay, RNAs were injected into the two dorsal blastomeres of 4-cell-stage embryos. Dorsal marginal-zone explants were dissected at early gastrula stage (stage 10) and incubated to mid-gastrula (stage 11) or tailbud (stage 28) stages. Xhox3, Wnt8 and globin are ventral markers, whereas OtxA and collagen II are dorsal markers. The weak induction of Wnt8 is not always observed. In **e**, the basic FGF protein (Sigma) was added at 100 ng ml^{-1} to the animal caps at the blastula stages. The amount of RNA injected into the embryos was: 2 ng, all Tsg; 0.5 ng, Bmp2; 0.5 ng, Bmp4; 5 pg, activin; and 50 pg, Wnt8.

To study the function of Tsgs, we first analysed their activities in *Xenopus* ectodermal explants (animal caps). As shown in Fig. 1a, human, mouse and *Xenopus* Tsg induce the cement gland and the neural markers XAG-1, OtxA and NRP-1 with comparable efficiency, suggesting that these vertebrate Tsgs function similarly in *Xenopus*. The induction of cement gland and neural markers in animal caps in the absence of mesoderm is normally associated with inhibition of the BMP signalling[16–18], so we therefore addressed whether Tsg could directly block the activity of BMP. We first examined the effect of Tsg on ventralization of the ectodermal cells by BMPs. As described previously[16], intact animal caps express high levels of epidermal keratin. This expression is suppressed when caps from blastula stages are dissociated for 4 h (Fig. 1b, lanes 1 and

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The Developmentally Regulated Expression of *Twisted Gastrulation* Reveals a Role for Bone Morphogenetic Proteins in the Control of T Cell Development

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Abstract

The evolutionarily conserved, secreted protein Twisted gastrulation (Tsg) modulates morphogenetic effects of decapentaplegic (dpp) and its orthologs, the bone morphogenetic proteins 2 and 4 (BMP2/4), in early *Drosophila* and vertebrate embryos. We have uncovered a role for Tsg at a much later stage of mammalian development, during T cell differentiation in the thymus. BMP4 is expressed by thymic stroma and inhibits the proliferation of $CD4^-CD8^-$ double-negative (DN) thymocytes and their differentiation to the $CD4^+CD8^+$ double-positive (DP) stage in vitro. *Tsg* is expressed by thymocytes and up-regulated after T cell receptor signaling at two developmental checkpoints, the transition from the DN to the DP and from the DP to the $CD4^+$ or $CD8^+$ single-positive stage. Tsg can synergize with the BMP inhibitor chordin to block the BMP4–mediated inhibition of thymocyte proliferation and differentiation. These data suggest that the developmentally regulated expression of *Tsg* may allow thymocytes to temporarily withdraw from inhibitory BMP signals.

Key words: BMP4 • Twisted gastrulation • thymocyte development • chordin • morphogen

Introduction

Thymocyte differentiation is controlled by signals via the pre-TCR and the TCR (for reviews, see references 1–3). Progression from the $CD4^-CD8^-$ double-negative (DN)* to the $CD4^+CD8^+$ double-positive (DP) stage in cells destined for the TCR αβ lineage is driven by pre-TCR signaling and requires TCRβ chain rearrangement, expression, and pairing with the pre-TCR α chain. The acquisition of CD4 and CD8 is accompanied by a burst of proliferation, the cessation of further V to DJ rearrangement at the TCR β locus and proceeds through a transitional stage where (in the mouse strains used in this study) CD8 is expressed before CD4 (1). Recombination-deficient thymocytes are blocked at the $CD4^-CD8^-CD25^+CD44^-$ stage of development but antibodies to the CD3ε signaling chain can mimic pre-TCR signaling and trigger progression to the DP stage (2). Further development from the DP to the $CD4^+$ or $CD8^+$ single-positive (SP) stage of development requires rearrangement and pairing of TCRα with TCRβ and the engagement of the resulting heterodimer with suitable peptide/MHC ligands on thymic stromal cells (3).

The control of cellular differentiation by antigen receptor rearrangement, expression, and engagement is unique to lymphocytes and (in evolutionary terms) a recent addition to older systems comprising soluble and cell-associated factors that control the survival, growth, and differentiation of cells and tissues in all multicellular organisms. For example, thymocyte survival and differentiation are promoted by cytokines including IL-7 (4), c-Kit ligand (5), IL-1, and TNF (6), and signaling through Notch (7) participates in T cell lineage commitment (8). Comparatively little is known about the role of patterning molecules or morphogens in lymphocyte development (9–12).

In a screen for genes that are regulated in a developmental stage-specific manner in thymocytes (13) we have identified *Twisted gastrulation* (*Tsg*; reference 14). Recent studies have revealed that the Tsg protein interacts with *Drosophila*

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**Abbreviations used in this paper:* BMP, bone morphogenetic protein; DN, double negative; DP, double positive; dpp, decapentaplegic; pSmad, phospho-Smad; Rag, recombination activating gene; RT, reverse transcription; sog, short gastrulation; SP, single positive; Tsg, twisted gastrulation.

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decapentaplegic (dpp), the vertebrate dpp orthologs bone morphogenetic protein (BMP)2/4, and also the extracellular dpp/BMP inhibitors short gastrulation (sog)/chordin (15–20). In addition, Tsg can alter the proteolytic processing of sog/chordin by extracellular metalloproteases (16, 20). As a result, Tsg affects the binding of dpp/BMP2/4 to their cellular receptors and downstream signaling events mediated by the phosphorylation, nuclear translocation, and transcriptional activity of Smad proteins (for a review, see reference 21) positively (15, 20) or negatively (16–20). BMPs belong to a family of secreted signaling molecules the founding member of which, TGFβ, is essential for immune homeostasis (11, 22). In addition to a well-established role in embryonic patterning and development (23), BMP4 has been linked to hematopoesis: it specifies ventral mesoderm and blood cell formation in the *Xenopus* embryo (24), cooperates with VEGF to enhance hematopoetic cell generation from ES cells (25), is expressed in the human fetal AGM region (26), and regulates primitive human hematopoetic cell proliferation (27). There is evidence that components of the BMP signaling pathway are expressed in the thymus, including BMP4 itself (9), the extracellular BMP inhibitor chordin (28), the BMP receptor components activin-like kinase (ALK)-3 and -6 (BMPR1A and -B; reference 29), and Smad proteins (30, 31), the downstream mediators of BMP signaling. However, a role for BMP signals in thymocyte development has not been described. Furthermore, our understanding of *Tsg* is currently limited to early embryonic development (15–20, 32). We have investigated the effects of BMP4 and its modulation by Tsg during the transition from the $CD4^-CD8^-$ DN to the $CD4^+CD8^+$ DP stage of thymocyte development.

Materials and Methods

Mouse Strains, Cell Sorting, Cell and Organ Culture. Thymi were derived from wild-type (BALB/c or C57BL/6), recombination activating gene (Rag)$1^{o/o}$ (33), or $A\beta^{o/o}$ $\beta2m^{o/o}$ (34, 35; referred to as $MHC^{o/o}$ in this manuscript). Where indicated, 3–4-wk-old Rag-$1^{o/o}$ mice were injected with 50 μg of the CD3ε mAb 2C11 (BD PharMingen). Thymocyte organ cultures and suspension cultures of mechanically dissociated or trypsinized fetal thymi were set up as hanging drops in inverted Terasaki plates (Nunc) in serum-free AIM-V lymphocyte medium (GIBCO BRL) supplemented with 2×10^{-5} M 2-ME, where indicated in the presence of recombinant BMP2, -4, and -7, chordin, neutralizing anti-BMP4 or BMPR-IA/Fc (all from R&D Systems), TGFβ1 (Sigma-Aldrich), or the CD3ε antibody 2C11 (BD PharMingen). Recombinant mouse Tsg was produced in X63 myeloma cells transfected with full-length mTsg cDNA (14) tagged with a COOH-terminal HA epitope and inserted into the BCMGS neo vector (36). Supernatant was concentrated, mTsg-HA captured with anti-HA–conjugated agarose beads (Sigma-Aldrich) and eluted by incubation of the washed beads with 100 μg/ml HA peptide (Sigma-Aldrich). Western analysis of the material with an anti-HA antibody (Sigma-Aldrich) showed a single band at ~25 kD. Tsg concentration was estimated by gel staining since the HA peptide used for elution interfered with measurement of protein concentration. Supernatant from X63 cells transfected with empty BCMGS neo vector treated in the same way served as a control for mTsg-HA. All Tsg effects were confirmed using commercial mTsg (R&D Systems) which became available during the course of this study.

For some experiments thymocytes were stained with biotinylated antibodies to CD4 or CD8 and depleted with streptavidin-coated paramagnetic beads (Dynal). Thymic stromal cells were prepared by trypsinization of deoxyguanosine-treated fetal thymi and centrifugation over 55% Percoll (Amersham Pharmacia Biotech). Wild-type thymi were cultured for 18 to 72 h, thymus cell suspensions and thymocytes for 18 h, and Rag$1^{o/o}$ thymi for 48–72 h in the presence of the 2C11 mAb at 1 μg/ml. For phenotypic analysis on a FACSCalibur™ (Becton Dickinson) cells were counted and stained with CD4-PE or Cy-5 and CD8-FITC or -Cy5 (Caltag Laboratories), fixed for 10 min at room temperature in 0.2% paraformaldehyde, permeabilized with 0.2% Tween-20 for 15 min at 37°C, and incubated with 7-amino actinomycin D (7AAD). For sorting of DN and CD8 transitional cells on a FACS Vantage™ (Becton Dickinson), thymocytes were stained with CD4-Tricolor, CD8-PE (Caltag Laboratories), and heat stable antigen (HSA)-FITC.

Immunohistochemistry and RNA In Situ Hybridization on Frozen Sections. Thymi from 3–4-wk-old C57BL/6 mice were snap frozen, 6-μm sections were prepared. For immunohistochemistry, sections were fixed in acetone and stained using polyclonal goat anti-BMP2/4 (R&D Systems) followed by horseradish peroxidase–conjugated rabbit anti–goat Ig antibody (Dako) and liquid DAB substrate-chromogen solution (Dako) according to the manufacturer's instructions. Slides were counterstained with Mayer's Hematoxylin (Sigma-Aldrich) and mounted in Kaiser's solution (14). Sense and antisense riboprobes were synthesized from a cloned PCR fragment spanning exon 4 of mTsg (14) in the presence of 11-dUTP digoxigenin (Roche) with T3 or T7 RNA polymerase. After hybridization, high-stringency washing, and RNase A digestion, the sections were incubated with alkaline phosphatase–conjugated anti-digoxigenin (Roche). BCIP/NBT (Roche) was used as substrate for detection. The sections were counterstained with nuclear fast red (Vector Laboratories), mounted in Mowiol solution (Calbiochem), and photographed.

Molecular Cloning and Analysis. Expression studies were performed as reported previously (14, 37). For Northern blots, 1–5 μg total RNA were separated on 1% agarose gels containing formamide. The RNA was transferred to HybondN+ Nylon membranes and hybridized in 1.5× SSPE, 10% PEG 8000, 7% SDS at 68°C overnight. After washing in decreasing SSC concentrations and a final stringency wash in 0.1× SSC, 0.5% SDS at 60°C the membranes were exposed to BioMax-MS film (Eastman Kodak Co.).

For reverse transcription (RT)-PCR experiments, 0.3–1 μg total RNA were reverse transcribed with AMV reverse transcriptase (Promega) and amplified with the following primers at annealing temperatures of 60°C to 63°C: BMP2 no. 465 TACCGCAGGCACTCAGG, no. 466 CATTCCACCCCACATCACT; BMP4 no. 467 CGAGGCGACACTTCTACAG, no. 468 TGGGGGCTTCATAACCT; chordin: no. 473 CAAGCCTCAGCGGAAGAA, no. 474 CAAGCCCAGCCAATAGAACT; GAPDH: no. 251 TCTTCTTGTGCAGTGCC, no. 252 ACTCCACGACATACTCAGC. PCR products were analyzed on 2% agarose gels and the identity of the bands was confirmed by blotting and hybridizing with specific probes.

Western Blotting. Phospho-Smad (pSmad)-2–specific antibodies were used to assess the phosphorylation status of Smad-2. Thymocyte lysates were separated (2×10^6 cell equivalents per lane) on 10% SDS polyacrylamide gels and blotted onto PVDF

membranes (NEN Life Science Products). Blots were blocked (1% nonfat milk in PBS, 0.1% Tween 20 for p-Smad-2; 5% nonfat milk in PBS) and probed with rabbit anti-pSmad2 (Upstate Biotechnology 06–829) followed by alkaline phosphatase–conjugated anti–rabbit IgG and enhanced chemoluminescence detection (Western Star; Tropix) on MR-1 film (Eastman Kodak Co.). Blots were stripped and reprobed for total Smad-2 and subsequently for lamin B to control for equal loading and transfer efficiency (not shown).

Results

Tsg Up-regulation After TCR Signaling at Control Points in Thymocyte Development. We undertook a screen to find novel genes involved in T cell development. MHC-naive $CD4^+CD8^+$ DP thymocytes (isolated from $MHC^{o/o}$ mice) were cocultured with thymic stromal cells derived from MHC-expressing (wild-type) or, as a control, from $MHC^{o/o}$ thymi (37). This system allowed a comparison of gene expression profiles between MHC-exposed and MHC-naive thymocytes by differential display PCR (13). The characterization of a band consistently up-regulated upon TCR signaling resulted in the cloning of the mouse homologue of *Tsg* (14). Northern blotting confirmed increased *Tsg* expression by MHC-naive DP thymocytes between 6 and 24 h after treatment with antibodies to the TCR alone or in combination with anti-CD28 (Fig. 1 a). To investigate *Tsg* expression at the pre-TCR–driven transition from the $CD4^-CD8^-$ DN to the $CD4^+CD8^+$ DP stage we injected $Rag1^{o/o}$ mice with antibodies to CD3ε. Thymocyte development in $Rag1^{o/o}$ mice is blocked at the $CD44^-CD25^+$ DN stage of differentiation and can be rescued by CD3ε treatment in vivo which triggers a synchronous wave of differentiation from the $CD44^-CD25^+$ DN to the DP stage (2). This was accompanied by a rise in *Tsg* expression that occurred around 48 h after anti-CD3 injection (Fig. 1 b), at a time when thymocytes showed reduced CD25 expression and increased proliferation but were still DN (reference 2, and data not shown). We conclude that *Tsg* is differentially expressed after TCR signaling at two developmental checkpoints, the transition from the $CD4^-CD8^-$ DN to the $CD4^+CD8^+$ DP and from the DP to the $CD4^+$ or $CD8^+$ SP stage.

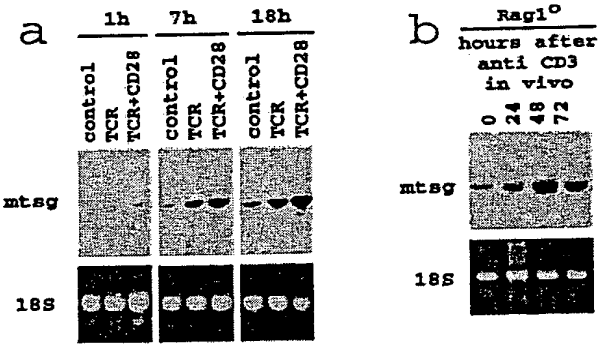

Figure 1. TCR-regulated Tsg expression by thymocytes. (a) Increased *Tsg* mRNA expression by MHC-naive DP thymocytes in response to antibodies to the TCR, with or without CD28 engagement. (b) Increased *Tsg* mRNA expression by DN thymocytes 48 h after injection of $Rag\text{-}1^{o/o}$ mice with anti-CD3ε. In additional experiments, *Tsg* upregulation occurred between 24 and 72 h and persisted until 120 h after injection.

The recognized role of Tsg as a modulator of BMP signaling (15–20) prompted us to reevaluate the expression of components of the BMP signaling pathway in the thymus (see Introduction). RT-PCR showed the expression of BMP2, BMP4, BMP7, and chordin in total thymus tissue (Fig. 2 a). Analysis of separated thymocytes and thymic stroma established thymic stromal cells as the source of BMP2/4 and chordin. BMP7 was expressed by both thymocytes and stromal cells (Fig. 2 a). Semiquantitative RT-PCR analysis confirmed that thymocytes expressed BMP7 at levels similar to total thymus whereas the levels of BMP4 and chordin expression in thymocytes were 20- to 100-fold lower in thymocytes than total thymus (data not shown). The distribution of BMP2/4 protein in the thymus was visualized by immunohistochemistry on frozen sections (Fig. 2, b–e). We noted marked regional differences with staining predominantly of subcapsular (sc) and medullary (med) regions. Within these areas the signal appeared 'patchy' rather than uniform (Fig. 2, c and e). In situ hybridization showed *Tsg* expressing cells distributed throughout the cortex and the medulla (Fig. 2, f–h).

BMP4 Can Interfere with Cell Cycle Activity and Developmental Progression of DN Thymocytes. To assess the functional effects of BMPs we established thymic organ cultures at embryonic day 15.5 (E15.5), a time in ontogeny when all thymocytes are DN, and analyzed the cell cycle distribution as well as the phenotype of thymocytes that developed in these cultures. Addition of BMP4 at 100 ng/ml (~6 nM) reduced the percentage of thymocytes in S and G2/M phase of the cell cycle by 42 ± 9% (Fig. 3, a and b). This inhibition was largely abrogated by a neutralizing antibody to BMP4 (Fig. 3 a). Conversely, treatment of thymic organ cultures with recombinant chordin 2 μg/ml (~20 nM) increased the percentage of thymocytes in S and G2/M by 59 ± 30% (Fig. 3 a). As chordin is an extracellular inhibitor specifically of BMPs but not of TGFβ or activin (21), the increased proliferation of thymocytes in intact thymic lobes treated with chordin suggests that thymocyte cell cycle activity is subject to BMP-mediated inhibition in situ.

In addition to proliferation, BMP4 treatment of E15.5 thymic organ cultures interfered with the developmental progression from the $CD4^-CD8^-$ DN to the $CD4^+CD8^+$ DP stage (Fig. 3 b) and reduced the generation of DP cells on average by 52 ± 21% (Fig. 3 c). In control experiments, the survival of sorted E17 DP thymocytes was unaffected by overnight culture in BMP4 (100 ng/ml), indicating that BMP4 was not simply toxic to DP thymocytes (data not shown).

The reduced generation of DP thymocytes in the presence of BMP4 could be explained either by a delay in TCRβ chain rearrangement or by a reduced response to pre-TCR signaling. To address this issue we assayed BMP4

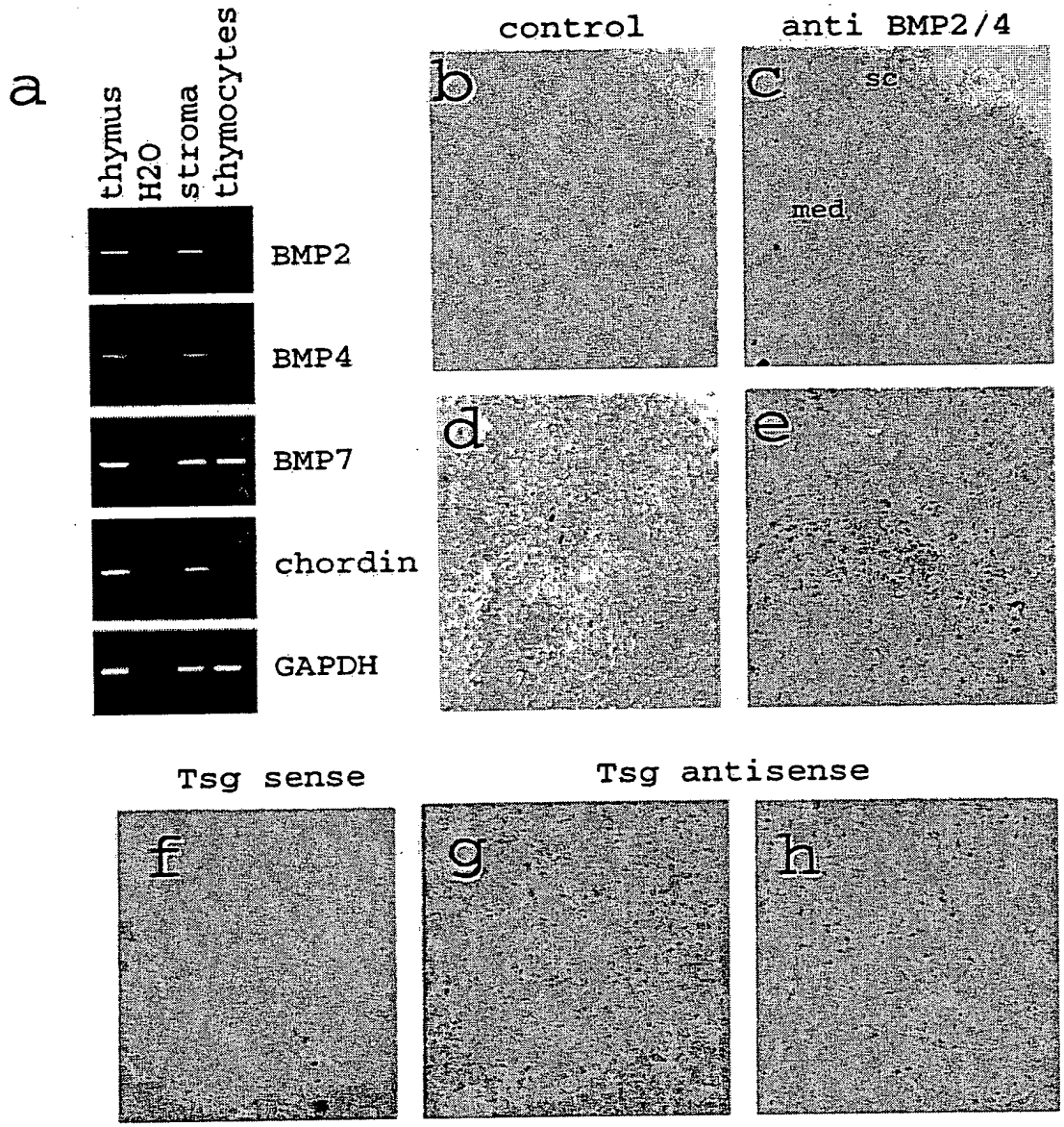

Figure 2. Expression and distribution of intrathymic BMPs and chordin. (a) Total thymus or isolated stromal cells and thymocytes were subjected to RT-PCR analysis to establish the sources of BMP2, BMP4, BMP7, and chordin in the thymus. GAPDH served as a positive control. H_2O lanes contained no cDNA. (b–e) Immunoperoxidase staining of normal 3–4-wk-old mouse (C57BL/6) thymus with goat anti-BMP/24 (c and e) or control goat antibody (b and d). BMP2/4 staining (brown) is seen mainly within supcapsular (sc) and medullary (med, outlined in c) regions (original magnification: ×200, b and c) and 'patchy' within positive areas (original magnification: ×400, d and e). (f–h) In situ hybridization of normal 3–4-wk-old mouse (C57BL/6) thymus with Tsg sense (f) and antisense (g and h) probes. *Tsg* expressing cells are distributed throughout the cortex and the medulla. Original magnification: ×100 (f and g), ×200 (h).

effects on the differentiation of Rag1-deficient thymocytes in response to CD3ε antibody (1 μg/ml) as a surrogate signal which does not require TCRβ chain rearrangement. BMP4 treatment reduced the development of DP thymocytes in $Rag1^{o/o}$ organ cultures treated with CD3ε antibodies by 46 ± 22% (Fig. 3 c).

To ask whether BMP4 inhibition of the DN to DP transition required an intact thymic microenvironment, E15.5 thymi were disrupted by trypsinization to yield suspensions containing all thymic cell types including thymic stroma. BMP4 blocked the DN to DP transition by 52 ± 17%. Similarly, BMP4 blocked the DN to DP transition in me-

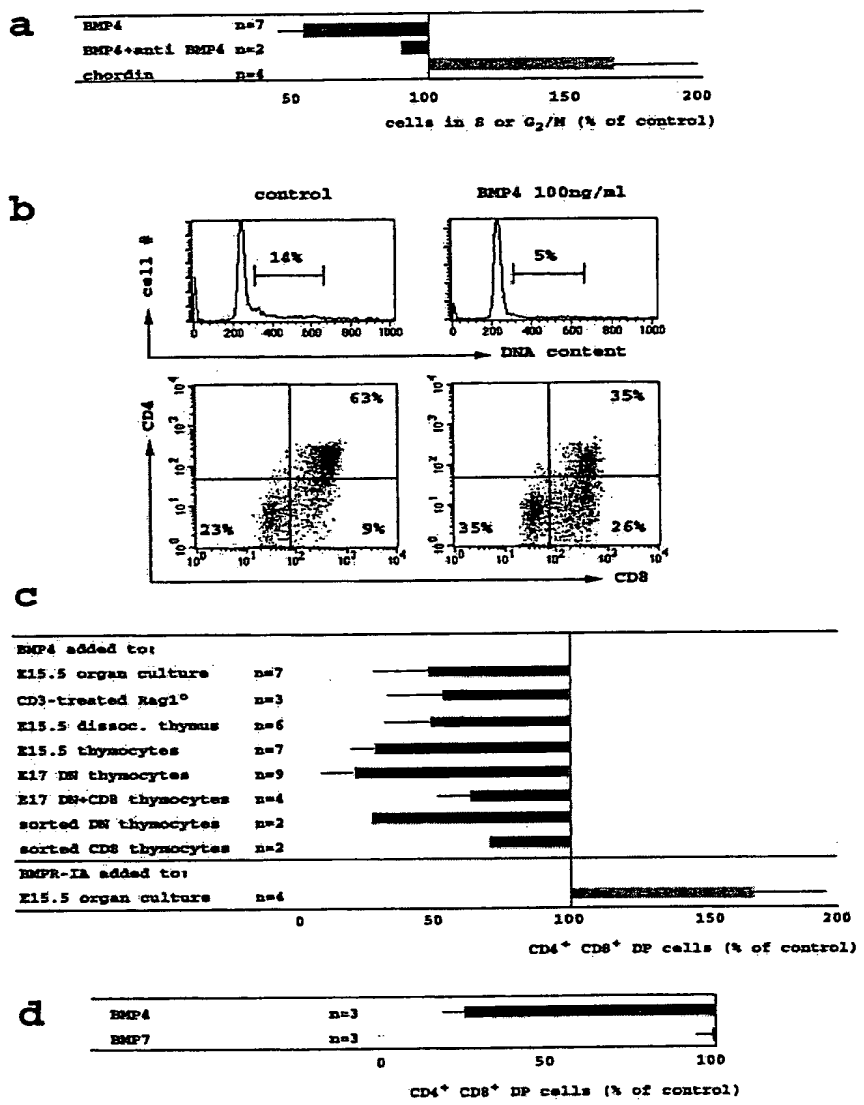

Figure 3. BMP4 inhibits the cell cycle activity and the developmental progression of DN thymocytes to the DP stage. (a) E15.5 thymic lobes were cultured in hanging drops for 24–36 h with BMP4 (100 ng/ml, 6 nM approximately), BMP4 (100 ng/ml) plus neutralizing anti-BMP4 (10 μg/ml) or chordin (2 μg/ml, 20 nM approximately). Thymocytes were stained for CD4 and CD8, fixed, permeabilized, and DNA content was visualized by 7AAD. The fraction of cells with a DNA content >G1 is shown relative to control cultures (16 ± 7% >G1). (b) Reduced cell cycle activity and impaired developmental progression in BMP4-treated E15.5 thymic organ cultures (36 h, methods as in a). (c) BMP4 (100 ng/ml) inhibited the DN to DP transition in E15.5 thymic organ cultures by 52 ± 21% (from 50 ± 10% DP to 25 ± 13% at 36 h, top row) and in Rag1$^{o/o}$ thymic organ cultures treated for 72 h with anti-CD3ε (1 μg/ml) by 46 ± 22% (from 57 ± 4% DP to 32 ± 12%, row two). BMP4 reduced the generation of DP cells in proteolytically dissociated E15.5 thymus suspensions by 52 ± 17% (from 59 ± 13% DP to 28 ± 9%, third row), in mechanically prepared E15.5 thymocyte suspensions by 72 ± 8% (from 44 ± 19% DP to 13 ± 6%, row four), in DN cells prepared by CD8 depletion of E17 thymocytes by 80 ± 13% (from 33 ± 16% to 8 ± 8%, row five), in DN/CD8 transitional thymocytes prepared by CD4 depletion of E17 thymocytes by 36 ± 10% (from 66 ± 23% DP to 44 ± 20%, row six), in highly purified DN by 72% (from 39% DP to 11%) and 73% (from 40% DP to 10%) in two experiments (row seven), and in highly purified CD8 transitional thymocytes by 26% (from 91% DP to 67%) and 29%, (from 83% DP to 59%) in two experiments (row eight, all suspension culture experiments read out at 18 h). Recombinant BMPR-1A/Fc (bottom row, 1–3 μg/ml) increased the generation of DP cells in E15.5 thymic lobes cultured for 18 h by 67 ± 28% (from 23 to 38%, n = 4). (d) E15.5 thymus suspensions were cultured with 300 ng/ml of BMP4 or BMP7 and analyzed as in Fig. 3, b and c. BMP4 reduced the generation of DP cells by 73 ± 7% (from 50 ± 27% DP to 14 ± 9%) but BMP7 did not (1 ± 5%).

chanically prepared E15.5 thymocyte suspensions by 72 ± 8% (Fig. 3 c).

The ability of BMP4 to inhibit thymocyte differentiation in suspension cultures allowed us to define the BMP4 sensitivity of thymocytes at different developmental stages and, in addition, to ask whether BMP4 acts directly on thymocytes or via intermediate cell types. When E17 thymocyte suspensions were depleted of CD8$^+$ thymocytes to yield pure DN cells, BMP4 inhibited the generation of DP cells by 80 ± 13%. In contrast, when E17 thymocyte suspensions were depleted only of CD4$^+$ thymocytes, leaving behind DN as well as CD8 transitional cells, BMP4 was less effective at inhibiting the generation of DP cells (36 ± 10%). Hence, DN thymocytes were more sensitive to BMP4 than CD8 transitional cells (Fig. 3 c).

To determine whether BMP4 affects thymocyte development directly or indirectly, DN thymocytes were highly purified by two rounds of cell sorting for HSA^{high}CD4$^-$CD8$^-$ thymocytes, yielding populations that contained 0.3% or less of cell types other than thymocytes. Sorted thymocytes differentiated efficiently to the DP stage and BMP4 inhibited this by 72% and 73% in two independent experiments. The developmental progression of highly purified CD8 transitional cells was blocked by BMP4 to a lesser degree, by 26 and 29% in two independent experiments (Fig. 3 c). Hence BMP4 acts directly on DN thymocytes to inhibit their progression to the DP stage.

We exploited the observation that BMP2 and 4 (and to a lesser extent BMP7) but not TGFβ or activin can bind BMPR-IA with high affinity in the absence of type II receptor subunits (38, 39) to address whether the DN to DP transition is affected by endogenous BMPs. Soluble BMP receptor-IA subunit (BMPR-IA) was added to intact E15.5 thymic lobes in organ culture. In the presence of BMPR-IA, the percentage of DP thymocytes (analyzed 18 h later instead of 24–36 h in the organ culture experiments described above) was elevated by 67 ± 28% relative to control thymi (Fig. 3 c) and the number of DP cells per thymic lobe increased by 94 ± 57% (not shown).

Exogenous BMP2 blocked the DN to DP transition to the same extent as BMP4 (not shown). In contrast, BMP7 did not inhibit the DN to DP transition (Fig. 3 d), even at very high concentrations (1,000 ng/ml, not shown).

Smad-2 Phosphorylation in Response to TGFβ but Not BMP4 in Thymocytes. Like BMP4, TGFβ blocks thymocyte cell cycle activity and developmental progression (10, 40). To address the question whether thymocytes can distinguish BMP4 from TGFβ signals we analyzed the phosphorylation status of Smad-2, a downstream mediator of TGFβ signals (21) in thymocytes cultured with TGFβ1 at 1 ng/ml or a range of BMP4 concentrations from 0.1 to 1,000 ng/ml. Phosphorylated Smad-2 was readily detected in response to TGFβ but not to BMP4 by Western blotting with pSmad-2–specific antibodies (Fig. 4). Hence, BMP4 signaling does not appear to utilize the canonical TGFβ signaling pathway in thymocytes. An analysis of Smad-1 phosphorylation in response to exogenous BMP4 remained inconclusive as pSmad-1 appeared to be present in freshly isolated thymocytes (not shown, and see Discussion).

Tsg Synergizes with the BMP Inhibitor Chordin to Block BMP4 Effects on Thymocyte Differentiation. As described in Fig. 3 c, BMP4 inhibited the developmental progression from the DN to DP stage in Rag1$^{o/o}$ organ cultures treated with CD3ε. In the presence of BMP4 (100 ng/ml) the BMP inhibitor chordin restored the generation of DP cells only partially, even when added in threefold molar excess (2 μg/ml). We used this system to assess the functional effects of Tsg. On its own (not shown) or in combination with BMP4 (100 ng/ml), Tsg (1 μg/ml, ~40 nM) had little effect. In combination with chordin, however, TSG was able to reverse the inhibitory effects of BMP4 on the generation of DP thymocytes in anti-CD3ε–treated Rag1$^{o/o}$ organ cultures (Fig. 5 a, top panel). Rescue of DP differentiation from BMP4-mediated inhibition by chordin and Tsg was also seen in trypsinized suspension cultures of E15.5 thymi. Again, chordin antagonized BMP4 only partially, Tsg on its own was without effect, but the combination of chordin and Tsg rescued DP thymocyte development effectively (Fig. 5 a, bottom panel). A representative experiment is shown in Fig. 5 b.

The ratio between chordin and Tsg was found critical for BMP antagonism read out as secondary axis formation in zebrafish (19). We therefore performed experiments in which the concentrations of BMP4 (100 ng/ml) and chordin (2 μg/ml) were kept constant and Tsg was titrated over a wide range of concentrations, from 0.03 to 3,000 ng/ml (Fig. 5 b). Tsg addition resulted in a dose-dependent inhibition of the BMP4 block on the DN to DP transition. We conclude that Tsg can synergize with chordin to antagonize BMP4-mediated inhibition of the DN to DP transition. In this system there was no indication that Tsg had BMP-agonistic effects at any ratio of chordin and Tsg tested.

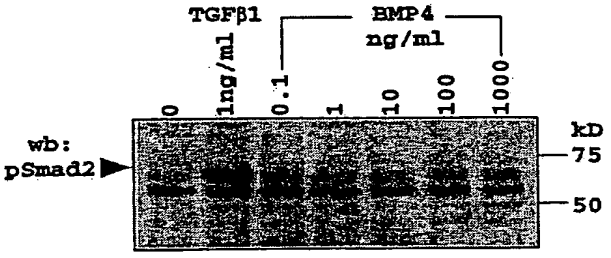

Figure 4. TGFβ but not BMP4 induce Smad-2 phosphorylation in thymocytes. Thymocytes from E17 (or postnatal, not shown) wild-type (C57BL/6) mice were cultured with TGFβ1 (1 ng/ml) or the indicated concentrations of BMP4 for 45 min and cell lysates assayed for pSmad-2 by Western blot.

Discussion

The key observation of our study is that immature thymocytes express Tsg, an extracellular modifier of BMP2/4 activity, in a developmentally regulated fashion: pre-TCR and TCR signaling results in increased Tsg expression at two developmental checkpoints, the DN to DP and the DP to SP transition (Fig. 1). Tsg has recently been shown to interact with extracellular components of the BMP signaling pathway (15–20), prompting us to investigate effects of BMP4 on thymocyte development. We found that DN thymocytes are highly susceptible to BMP4, which acts directly and without relay by another cell type to reduce DN thymocyte proliferation and progression to the DP stage in vitro (Fig. 3 c). The thymocyte response to BMP4 appears not to utilize the canonical TGFβ signaling cascade as indicated by the phosphorylation of Smad-2 in response to TGFβ but not to BMP4 (Fig. 4).

The addition of specific BMP inhibitors to intact thymic lobes results in elevated rates of thymocyte proliferation (Fig. 3 a) and accelerated pre-TCR dependent differentiation to the DP stage (Fig. 3 c), indicating that a BMP-imposed 'brake' may be active in situ. Conversely, exogenous BMP4 interferes with DN proliferation progression to the DP stage (Fig. 3, a–d), suggesting that endogenous BMP2/4 levels are subsaturating, or, alternatively, that not all thymocytes are exposed to equal BMP2/4 levels in situ. The latter possibility is consistent with our analysis of thymic sections where (in contrast to the uniform expression of BMP in early embryos; for a review, see reference 23) we found marked regional differences: BMP2/4 was seen predominantly in subcapsular and medullary areas and showed an uneven, 'patchy' distribution within these areas (Fig. 2, c and e). By analogy to the *Drosophila* ovary where the

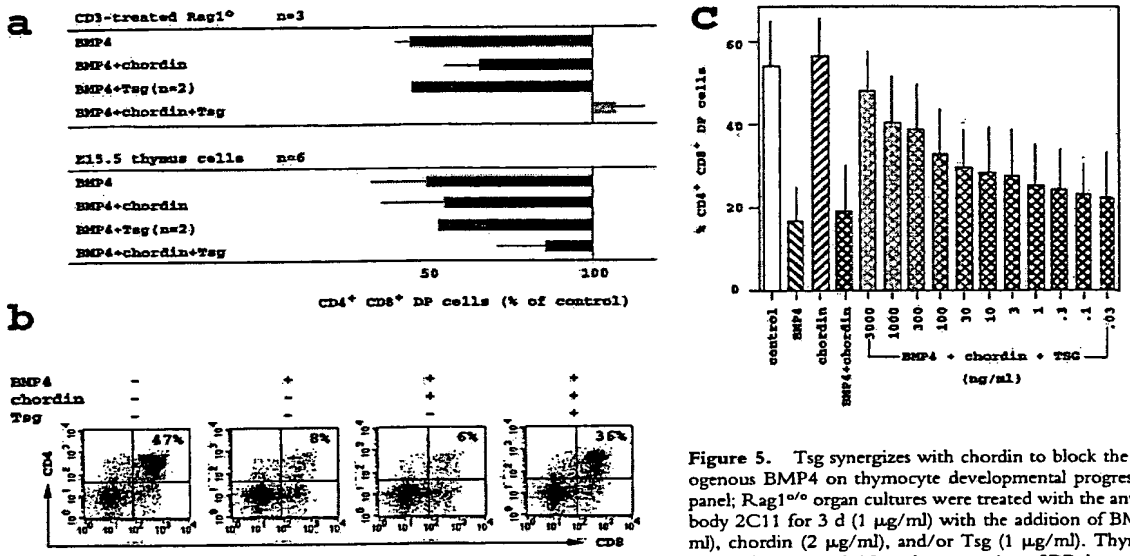

Figure 5. Tsg synergizes with chordin to block the effects of exogenous BMP4 on thymocyte developmental progression. (a) Top panel; Rag1$^{o/o}$ organ cultures were treated with the anti-CD3ε antibody 2C11 for 3 d (1 μg/ml) with the addition of BMP4 (100 ng/ml), chordin (2 μg/ml), and/or Tsg (1 μg/ml). Thymocytes were analyzed as in Fig. 3. Note the restoration of DP thymocyte development by the combination of chordin and Tsg. Bottom panel; suspension cultures of wild-type E15.5 thymi cultured overnight with the same additions as in top panel. (b) Suspension cultures of wild-type E15.5 thymi were cultured overnight and analyzed as in a. (c) E15.5 thymus suspensions were treated with BMP4 (100 ng/ml, left diagonal pattern), chordin (2 μg/ml, right diagonal pattern), or BMP4 plus chordin (cross-hatched) with or without Tsg (0.03 to 3,000 ng/ml) and analyzed as in Fig. 3. The percentage of DP thymocytes generated after 24 h is given (mean ± SD, $n = 4$).

BMP2/4 ortholog dpp serves to retain germ cell precursors in a concentration-dependent fashion (41) one might speculate that BMP2/4-rich areas represent specialized microenvironments geared toward the retention of precursor cells. In contrast to BMP2/4, the expression of BMP7 (which together with BMP5 and -6 forms the 60A subgroup of BMPs distinct from BMP2/4; reference 23) is not restricted to thymic stroma and BMP7 is expressed abundantly in thymocytes (Fig. 2 a). Interestingly, exogenous BMP7 did not inhibit the DN to DP transition (Fig. 3 d). Differences in the response to BMP2/4 and 7 have previously been seen in developing neurons (42, 43). Both BMP2/4 and BMP7 signaling involve Smad-1 (21, 23) and it is unclear how developing neurons (42, 43) or thymocytes (this study) discriminate between these BMPs. BMP7 may contribute to pSmad-1 seen in freshly isolated thymocytes (data not shown). In addition to BMP2/4 (this paper), TGFβ1 and the morphogen sonic hedgehog (Shh) can block the pre-TCR–dependent transition from the DN to the DP stage in vitro (9, 40). The relationship between BMP, Shh, and Wnt signals in the thymus remains to be defined. In certain developmental contexts Shh acts upstream of dpp/BMP2/4 (27, 41, 44–47). Conversely, Wnt and its downstream effectors (which can promote thymocyte differentiation; references 11 and 12) have been shown to block BMP4 expression in *Xenopus* embryos (48).

Reportedly, Tsg can either facilitate or antagonize dpp/BMP2/4 activity (15–20), perhaps because the release of dpp/BMP2/4 from the extracellular inhibitors sog/chordin is alternatively blocked (16) or facilitated (20) by the proteolytic processing of sog/chordin by metalloproteases of the tolloid/BMP1 family. The relative abundance of Tsg and chordin can be critical for whether Tsg acts as an agonist or an antagonist of dpp/BMP2/4 (17). In our own experiments Tsg synergized with chordin to antagonize BMP4 in a simple, dose-dependent manner (Fig. 5). If BMP2/4 originating from thymic stroma can block the expansion and differentiation of DN thymocytes in vivo, the developmentally regulated expression of Tsg could serve to temporarily antagonize inhibitory BMP effects following successful TCRβ rearrangement and pre-TCR expression

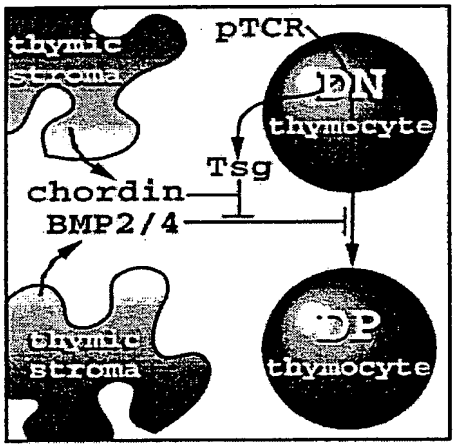

Figure 6. How BMP4, chordin, and Tsg may affect the pre-TCR–dependent DN to DP transition (see text for details).

(Fig. 6). We speculate that the balance between BMP2/4, chordin, and Tsg may ensure developmental progression while maintaining a sufficient pool of immature precursors. Although these ideas are consistent with our data on the developmental regulation of Tsg expression in vivo and our experiments in vitro, we emphasize that BMPs may have additional functions in the thymus. For example, a role at the DP stage is implied by differential Tsg expression in response to TCR engagement at the DP stage (Fig. 1) and the recently reported failure of thymocyte differentiate to the SP stage in the absence of Schnurri2, a putative downstream target of BMP signaling (49). Moreover, BMP4 and dpp act as morphogens in vertebrate and *Drosophila* embryos where they form activity gradients to specify distinct cell fates along the dorsal/ventral axis (23, 50–53). It will therefore be of interest to find out whether local BMP2/4 concentration specifies alternative fates such as $\alpha\beta$ versus $\gamma\delta$ at the DN to DP transition or CD4 versus CD8 at the DP to SP transition.

Our finding of developmentally regulated *Tsg* expression in the thymus extends the concept that cells within a morphogenetic field not only read and respond to the local morphogen concentration but can be instrumental in shaping the morphogen gradient (52, 53). It suggests that cells can temporarily withdraw from signaling molecules affecting their differentiation via the increased expression of a secreted modifier at specific developmental control points.

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IMMUNOBIOLOGY

THE IMMUNE SYSTEM IN HEALTH AND DISEASE

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Part I AN INTRODUCTION TO IMMUNOBIOLOGY

Chapter 1: Basic Concepts in Immunology

Chapter 2: The Induction, Measurement, and Manipulation of the Immune Response

Many microorganisms, especially bacteria, have conserved surface molecules that are recognized by phagocytic cells, which play an important part in the early elimination of infection as well as serving as professional antigen-presenting cells and thereby inducing the later adaptive immune responses. These phagocytic cells include macrophages and neutrophils, which not only ingest and destroy extracellular microorganisms, and in particular bacteria, but are also important in recruiting other cells and molecules of the immune system by releasing chemicals that have effects collectively called inflammation.

1-19 Infection often triggers an inflammatory response.

The term **inflammation** is purely descriptive and was originally defined by the four Latin words *dolor*, *rubor*, *calor*, and *tumor*, meaning pain, redness, heat, and swelling. These changes result from changes in the local blood vessels, leading to their dilation, increased permeability, and increased stickiness for passing leukocytes and lymphocytes. The increased blood flow accounts for the heat and redness, while the leakage of cells and fluids into the tissue and their local actions account for the pain and swelling. The main cell types seen in inflammatory responses are polymorphonuclear neutrophilic leukocytes together with macrophages and their precursor monocytes; these are therefore known as **inflammatory cells**. Lymphocytes, as well as small numbers of eosinophils and basophils, also accumulate at sites of inflammation and, when extreme vascular leakage occurs, red blood cells may also occasionally be found. Inflammatory responses can be triggered directly by pathogens, especially bacteria, early in infection, and may be sustained later by antibodies and by T cells, which release inflammatory factors. In the early phase of an infection, inflammatory responses are important in attracting nonspecific inflammatory cells such as monocytes and neutrophils to the site of an infection. Later, the same changes attract effector lymphocytes, and

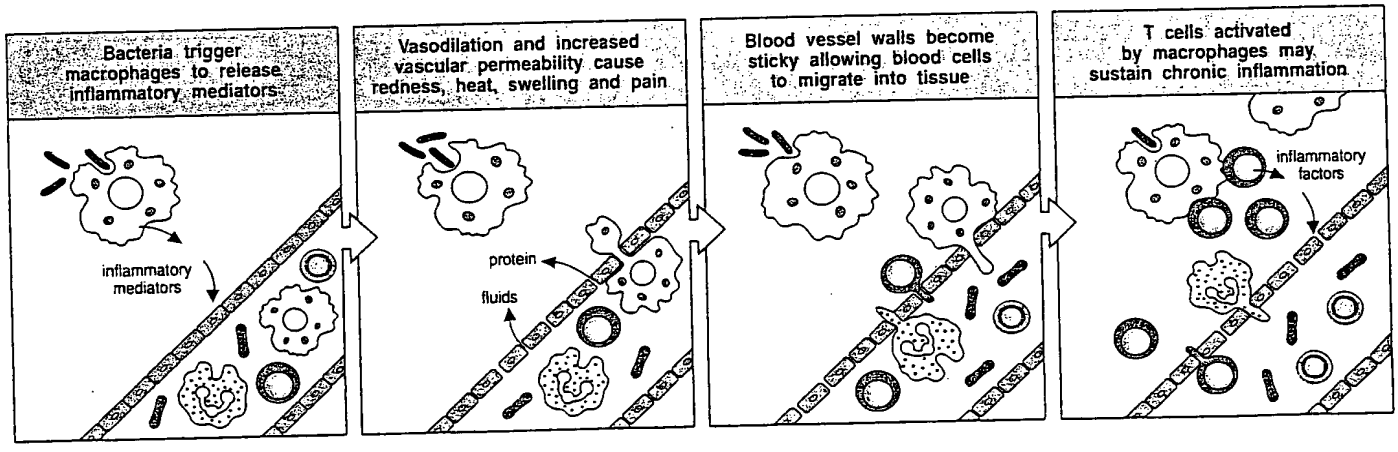

Fig. 1.32 Bacterial infection triggers an inflammatory response. Macrophages encountering bacteria in the tissues are triggered to release chemicals that increase the permeability of blood vessels, allowing fluid and proteins to pass into the tissues. The stickiness of the endothelial cells of the blood vessels is also changed, so that cells adhere to the blood vessel wall and are able to crawl through it: macrophages and polymorphonuclear neutrophilic leukocytes (neutrophils) are shown here entering the tissue from a blood vessel. The accumulation of fluid and cells at the site of infection causes the swelling, heat, and pain that are collectively known as inflammation. Macrophages and neutrophils are the principal inflammatory cells. Later in an immune response, activated lymphocytes also contribute to inflammation.

the increased permeability of the blood vessels allows the passage of antibodies into infected tissues (Fig. 1.32).

Many different stimuli can trigger inflammatory responses. Physical injury from wounds or burns releases proteins from tissues that trigger acute inflammatory reactions similar to those activated directly by bacteria. Chronic inflammatory processes are usually triggered by T cells, especially those that activate macrophages, as activated macrophages frequently cause local tissue damage through the release of mediators similar to those elicited by bacteria. Finally, some forms of acute inflammatory response are triggered by specific antibodies binding to antigen and activating the complement system, or interacting with accessory cells through their receptors for bound antibody molecules, as we shall see in the next section. The inflammatory response is a general term to describe both the gross and microscopic picture of local tissue infiltration by fluid and cells triggered in these different ways.

1-20 Specific recognition of pathogens by antibodies activates non-specific accessory cells.

Many microorganisms have evolved adaptations to their surface molecules that enable them to escape direct detection by any of the innate mechanisms we have described above. These microorganisms must be recognized by lymphocytes whose diverse receptors enable them to detect any pathogen and mount an adaptive immune response. The mechanisms whereby microorganisms are then destroyed, however, are essentially the same for the innate and adaptive arms of the immune response.

Thus, bacteria that resist direct binding by complement and are not bound by acute-phase proteins can become coated with specific antibodies. Once the antibodies have bound to the bacterium, they in turn recruit complement (see Fig. 1.22), as well as **accessory effector cells** that have receptors for bound antibody and complement molecules. These effector cells are the same as those that participate in innate immunity, and thus antibody, by flagging a pathogen as foreign, is able to overcome the ability of some pathogens to evade innate immune mechanisms. The accessory cells and the mechanisms whereby they eliminate pathogens are summarized in Fig. 1.33; we shall learn more about these cells when we discuss humoral immunity in Chapter 8.

Similarly, T cells recognize antigen specifically, but then trigger effector mechanisms that are not antigen specific. Specificity in cell-mediated immunity comes from the antigen-specific release of antigen-nonspecific effector molecules. Thus, killer CD8 T cells release their cytotoxic molecules only when they encounter an infected host cell, and inflammatory CD4 T cells activate only infected macrophages. Only in the case of B-cell activation by helper T cells is the target of T-cell action also antigen specific; however in this case, as we have just seen, the effector mechanism ultimately activated by the antibodies will not be antigen-specific. Thus, in both humoral and cell-mediated immunity, specificity derives from the clonally distributed receptors on antigen-specific lymphocytes, while effector function is mediated by cells and molecules that are not specific for antigen. This allows the same effector mechanisms to be used in response to a wide range of distinct pathogens.

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CELLULAR AND MOLECULAR IMMUNOLOGY

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CHAPTER ELEVEN

CYTOKINES

stimulates the growth of others. Often, TGF-β can either inhibit or stimulate growth of the same cell type, depending upon culture conditions such as degree of confluence. TGF-β causes synthesis of extracellular matrix proteins, such as collagens, and of cellular receptors for matrix proteins. (The ability of TGF-β to induce extracellular matrix probably underlies its ability to promote cell growth in soft agar.) *In vivo*, TGF-β causes the growth of new blood vessels, a process called angiogenesis.

As a cytokine, TGF-β is potentially important because it antagonizes many responses of lymphocytes. For example, TGF-β inhibits T cell proliferation to polyclonal mitogens or in mixed leukocyte reactions (see Chapter 16) and inhibits maturation of CTLs. It can also inhibit macrophage activation. TGF-β also acts on non-immune effector cells, such as polymorphonuclear leukocytes and endothelial cells, again largely to counteract the effects of pro-inflammatory cytokines. In this sense, TGF-β is an "anti-cytokine" and may be a signal for shutting off immune responses. Signals that cause T cells to synthesize TGF-β may cause them to behave as suppressor cells (see Chapter 10). *In vivo*, certain tumors may escape an immune response by secreting large quantities of TGF-β.

Although TGF-β is largely a negative regulator of immunity, it may have some positive effects as well. For example, in mice, TGF-β has been shown to switch B cells to the IgA isotype and it may therefore be important in the generation of mucosal immune responses that are mediated by IgA.

Cytokines That Activate Inflammatory Cells

We will now discuss a group of cytokines derived principally from antigen-activated $CD4^+$ and $CD8^+$ T lymphocytes that serve primarily to activate the functions of nonspecific effector cells. Thus, these cytokines mediate the effector phase of cell-mediated immune responses. The molecules described in this section are summarized in Table 11-3.

Immune or Gamma Interferon

Gamma interferon (IFN-γ), also called immune or type II interferon, is a homodimeric glycoprotein containing approximately 21 to 24 kD subunits. The size variation of the subunit is caused by variable degrees of glycosylation, but each subunit contains an identical 18 kD polypeptide encoded by the same gene. IFN-γ is produced both by IL-2 secreting $CD4^+$ helper T cells and by nearly all $CD8^+$ T cells. Transcription is directly initiated as a consequence of antigen activation and is enhanced by IL-2. IFN-γ has been detected in profoundly T cell-deficient animals, and the presumed source in this setting is NK cells; however, NK cells appear to be a minor source of IFN-γ in normal individuals.

As its name implies, IFN-γ shares many activities with type I IFN. Specifically, IFN-γ induces an antiviral state and is antiproliferative. However, IFN-γ binds to a unique cell surface receptor, different from that utilized by type I IFN. The IFN-γ receptor is not related structurally to the other receptor families described earlier. More importantly, IFN-γ has several properties related to immunoregulation that separate it functionally from type I IFN.

1. *IFN-γ is a potent activator of mononuclear phagocytes.* It directly induces synthesis of the enzymes that mediate the respiratory burst, allowing macrophages to kill phagocytosed microbes. Along with second signals, such as LPS and perhaps TNF, it allows macrophages to kill tumor cells. Cytokines that cause such functional changes in mononuclear phagocytes have been called **macrophage-activating factors** (MAFs). *IFN-γ is the principal MAF and provides the means by which T cells activate macrophages.* Other MAFs include GM-CSF and, to a lesser extent, IL-1, TNF, and, in the mouse, IL-4. Macrophage activation is described in more detail in Chapter 12. It is worth noting here that macrophage activation actually in-

TABLE 11-3. Mediators of Effector Cell Activation

Cytokine	Number of Genes	Polypeptide Size	Cell Source	Cell Target	Primary Effects on Each Target
Gamma interferon	1	21-24 kD (homodimer)	T cell, NK cell	Mononuclear phagocyte	Activation
				Endothelial cell	Activation
				NK cell	Activation
				All	Increased class I and class II MHC molecules
Lymphotoxin	1	24 kD (homotrimer)	T cell	Neutrophil	Activation
				Endothelial cell	Activation
Interleukin-5	1	20 kD (monomer)	T cell	Eosinophil	Activation
				B cell	Growth and activation
Migration inhibition factor	?	?	T cell	Mononuclear phagocyte	Conversion from motile to immotile state

Abbreviations: NK, natural killer; kD, kilodalton; MHC, major histocompatibility complex.

volves several different responses, and macrophages are said to be activated when they perform a particular function being assayed. For example, IFN-γ fully activates macrophages to kill phagocytosed microbes but only partly activates macrophages to kill tumor cells.

2. *IFN-γ increases class I MHC molecule expression and, in contrast to type I IFN, also causes a wide variety of cell types to express class II MHC molecules.* Thus, IFN-γ amplifies the cognitive phase of the immune response by promoting the activation of class II-restricted CD4+ helper T cells. *In vivo,* IFN-γ can enhance both cellular and humoral immune responses through these actions at the cognitive phase.

3. *IFN-γ acts directly on T and B lymphocytes to promote differentiation.* It is one of the factors that promotes CTL maturation and also stimulates B cell secretion of antibody. In mice, it causes B cell switching to the Ig2a isotype. IFN-γ is not a growth factor for lymphocytes and often inhibits proliferation of lymphocytes, particularly B cells. In mice, IFN-γ can antagonize IL-4 mediated effects, such as isotype switching to IgE.

4. *IFN-γ activates neutrophils,* upregulating their respiratory burst. It is a less potent activator of neutrophils than TNF or lymphotoxin.

5. *IFN-γ is a potent activator of NK cells,* more so than type I IFN.

6. *IFN-γ is an activator of vascular endothelial cells,* promoting CD4+ T lymphocyte adhesion and morphologic alterations that facilitate lymphocyte extravasation. As mentioned earlier, IFN-γ also potentiates many of the actions of TNF on endothelial cells.

LYMPHOTOXIN

Lymphotoxin is a 21 to 24 kD glycoprotein that is approximately 30 per cent homologous to TNF and competes with TNF for binding to the same cell surface receptors. In humans, LT and TNF genes are located in tandem within the MHC on chromosome 6 (see Chapter 5). LT is produced exclusively by activated T lymphocytes and is often produced coordinately with IFN-γ by such cells. Human LT, unlike TNF, contains one or two N-linked oligosaccharides (accounting for the variability in molecular sizes). In further contrast to TNF, LT is synthesized as a true secretory protein without a membrane-spanning region.

Most studies have found little difference between the biologic effects of TNF and LT, consistent with their binding to the same receptor. The most important distinction between these cytokines appears to be that LT is exclusively synthesized by T cells, whereas TNF, although made by T cells, is predominantly derived from mononuclear phagocytes. In general, the quantities of LT synthesized by T cells are much less than the amounts of TNF made by LPS-stimulated mononuclear phagocytes and LT is not readily detected in the circulation. Therefore, LT is usually a locally acting paracrine factor and not a mediator of systemic injury. Although neither TNF nor LT is toxic for normal (non-neoplastic) cells, both factors may contribute to CTL-mediated lysis of target cells (see Chapter 12). Like TNF, LT is a potent activator of neutrophils and thus provides lymphocytes with a means of regulating acute inflammatory reactions. It is more potent than IFN-γ as an activator of neutrophils and the actions of LT are enhanced by IFN-γ. LT is also an activator of vascular endothelial cells, causing increased leukocyte adhesion, cytokine production, and morphologic changes that facilitate leukocyte extravasation. These effects, like those of TNF, are also enhanced by IFN-γ.

INTERLEUKIN-5

Interleukin-5 (IL-5) is a cytokine of approximately 20 kD produced by certain activated CD4+ T cells and by activated mast cells. IL-5 acts as a costimulator for the growth of antigen-activated mouse B cells and was previously called either B cell growth factor 2 or T cell replacing factor. IL-5 may function synergistically with other cytokines, such as IL-2 and IL-4, to stimulate the growth and differentiation of B cells. IL-5 has also been found to act on more mature B cells to cause increased synthesis of immunoglobulin, especially of IgA. These actions are discussed in greater detail in Chapter 9.

An important action of IL-5 is its ability to stimulate the growth and differentiation of eosinophils and to activate mature eosinophils in such a way that they can kill helminths. In mice, neutralizing antibodies to IL-5 inhibit the eosinophilia seen in response to helminthic infection. Thus, IL-5 provides a means by which T cells can regulate eosinophil-mediated inflammation.

MIGRATION INHIBITION FACTOR

We conclude our discussion of cytokines that regulate effector cells by considering the issue of migration inhibitor factor (MIF). One early view of cell mediated immune reactions proposed that mononuclear phagocyte accumulation in tissues depended on the retention of such cells in response to locally produced cytokines that inhibit motility. It now seems more likely that retention of leukocytes in the tissues is controlled primarily by expression of specific receptors for extracellular matrix molecules, such as integrins (see Box 7-4, Chapter 7). Nevertheless, one of the first cytokine activities identified was one that inhibited macrophage motility *in vitro,* called migration inhibition factor. MIF has still not been identified as a unique cytokine, although some recently cloned molecules appear to demonstrate MIF activity. At present, both the biochemical identity and biologic signifiance of MIF remain largely undefined.

Cytokines That Stimulate Hematopoiesis

Several of the cytokines generated during both natural immunity and antigen-induced specific immune responses have potent stimulatory effects on the growth and differentiation of bone marrow pro-

CELLULAR AND MOLECULAR IMMUNOLOGY

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CHAPTER TWO

CELLS AND TISSUES OF THE IMMUNE SYSTEM

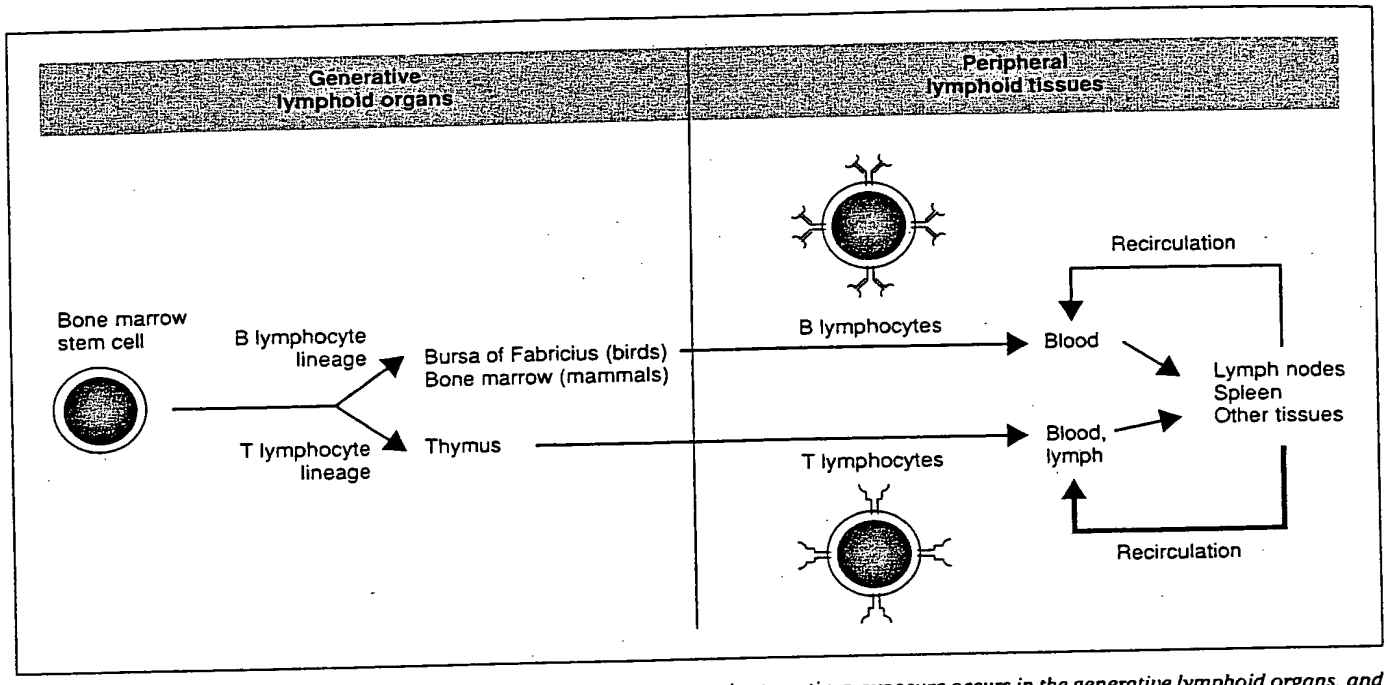

FIGURE 2-7. Maturation of lymphocytes. *Development of mature lymphocytes prior to antigen exposure occurs in the generative lymphoid organs, and immune responses to foreign antigens occur in the peripheral lymphoid tissues.*

brae, iliac bones, and ribs. The red marrow that is found in these bones consists of a sponge-like reticular framework located between bony trabeculae. The spaces in this framework are filled by fat cells and the precursors of blood cells, which mature and exit via the dense network of vascular sinuses to become part of the circulatory system.

All the blood cells originate from a common **stem cell** that becomes committed to differentiate along particular lineages, i.e., erythroid, megakaryocytic, granulocytic, monocytic, and lymphocytic (Fig. 2-8). Cytokines are known to stimulate the proliferation and maturation of various precursors. Since these growth factors are assayed by their ability to stimulate different types of leukocyte colonies to develop from marrow cells *in vitro*, they are called "colony-stimulating factors" (CSFs). Several of these cytokines are produced by T lymphocytes, including interleukin-3 (IL-3, also called multi-CSF), which acts on all stem cells, and granulocyte-monocyte-CSF (GM-CSF), which stimulates the formation of granulocytes and monocytes. Macrophages and marrow stromal cells produce GM-CSF and additional CSFs specific for granulocytes (G-CSF) or monocytes (M-CSF). Macrophages and stromal cells in the bone marrow produce two other cytokines, called interleukin-1 and interleukin-6, which further enhance colony formation by hematopoietic precursors in the presence of CSFs. A cytokine called interleukin-7, also produced by marrow stromal cells, has been shown to preferentially stimulate the maturation of B lymphocytes from marrow precursors. The properties and functions of these cytokines are described in Chapter 11. However, little is known about the nature of the uncommitted stem cell or the mechanisms that regulate its commitment to specific lineages. In 1988, techniques for reconstituting the immune system of congenitally immunodeficient mice with human lymphohematopoietic stem cells were described. These immunodeficient mice lack T and B lymphocytes, and after the implantation of human hematopoietic tissues, mature human T and B cells develop in the animals and populate the circulation and peripheral lymphoid tissues. Such approaches hold great promise for more precise identification and characterization of stem cells and their developmental pathways.

Thymus

The thymus is a bilobed organ situated in the anterior mediastinum. Each lobe is divided into multiple lobules by fibrous septa, and each lobule consists of an outer cortex and an inner medulla (Fig. 2-9). The cortex contains a dense collection of T lymphocytes, and the lighter-staining medulla is more sparsely populated with lymphocytes. Scattered throughout the thymus are non-lymphoid epithelial cells, which have abundant cytoplasm, as well as bone marrow-derived dendritic cells and macrophages. In the medulla are structures called **Hassall's corpuscles,** which are composed of tightly packed whorls of epithelial cells that may be remnants of degenerating cells. The thymus has a rich vascular supply and efferent lymphatic vessels that drain into mediastinal lymph nodes.

The lymphocytes in the thymus, also called **thymocytes,** are T lymphocytes at various stages of matu-

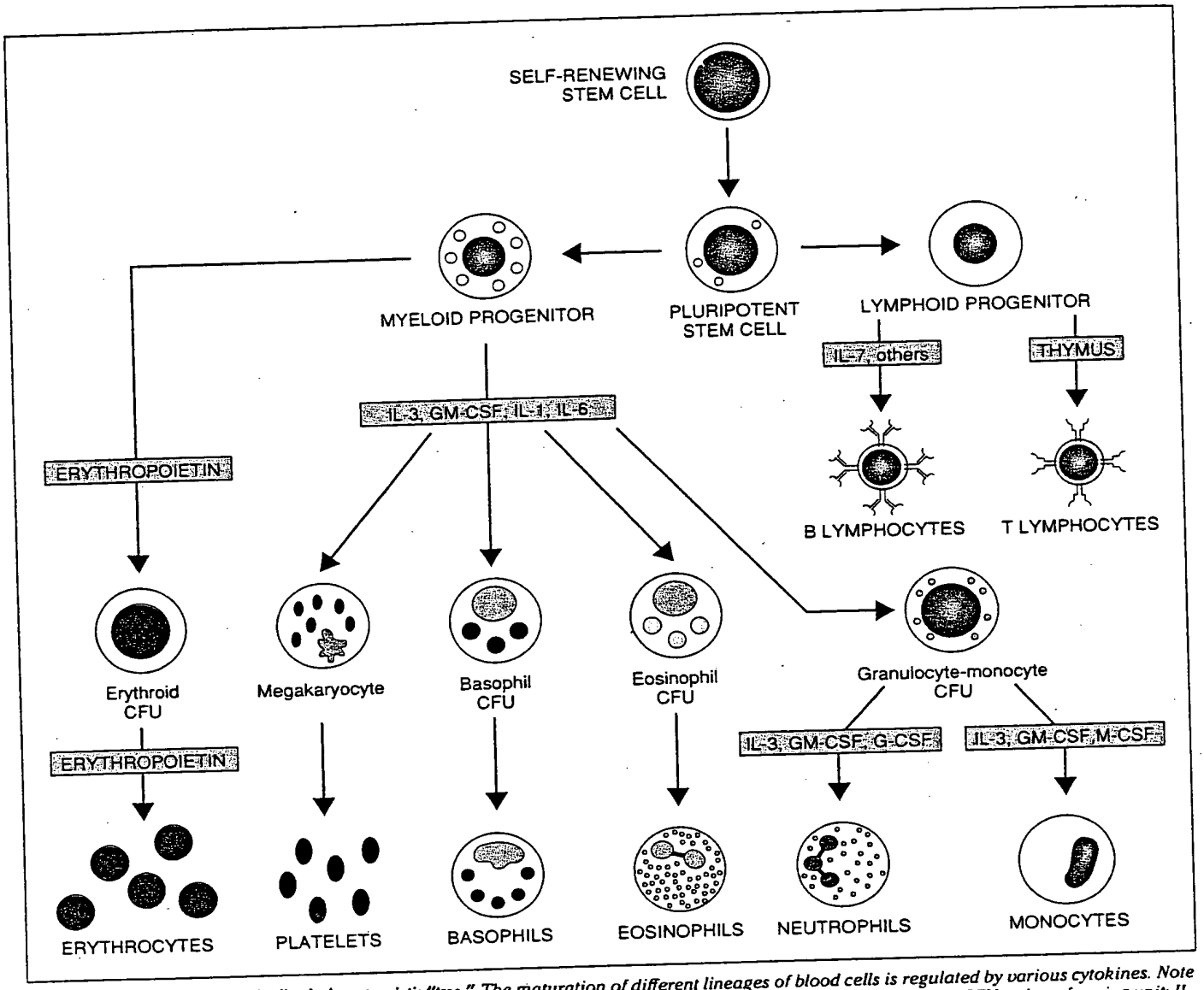

FIGURE 2 – 8. Maturation of blood cells: the hematopoietic "tree." *The maturation of different lineages of blood cells is regulated by various cytokines. Note that the maturation of T and B lymphocytes is illustrated in summary form and is discussed in detail in later chapters. CFU, colony-forming unit; IL, interleukin; GM - CSF, granulocyte-macrophage colony-stimulating factor.*

ration. Precursors that are committed to the T cell lineage enter the thymic cortex via blood vessels. It is not known whether B cell precursors enter the thymus and fail to survive or whether there are mechanisms that ensure that only cells committed to developing into T lymphocytes can enter the thymus. The most immature thymocytes do not express receptors for antigens or surface markers, including CD4 and CD8, that are characteristic of the mature phenotype. These immature cells migrate from the cortex toward the medulla and come into contact with epithelial cells, macrophages, and dendritic cells. Efficient contact may occur in lymphoepithelial complexes in which lymphocytes are found closely apposed to the invaginated plasma membranes of large epithelial cells called **"nurse cells."** En route to the medulla, thymocytes begin to express receptors for antigens and surface markers that are present on mature, peripheral T lymphocytes. Thus, the medulla contains mostly mature T cells, and only mature $CD4^+$ or $CD8^+$ T cells exit the thymus and enter the blood, lymph, and peripheral lymphoid tissues.

From the large number of primitive T cells that enter the thymus, cells that might recognize self antigens do not survive, whereas cells whose receptors are specific for foreign antigens are stimulated to mature. These selection processes, which are critical for the ability of the immune system to discriminate between self and non-self, are described in considerable detail in Chapter 8. The thymus is the site of remarkable proliferation as well as elimination of lymphocytes, which presumably reflect the selection of foreign antigen-specific cells and the deletion of potentially self-reactive T cells. It is estimated that in

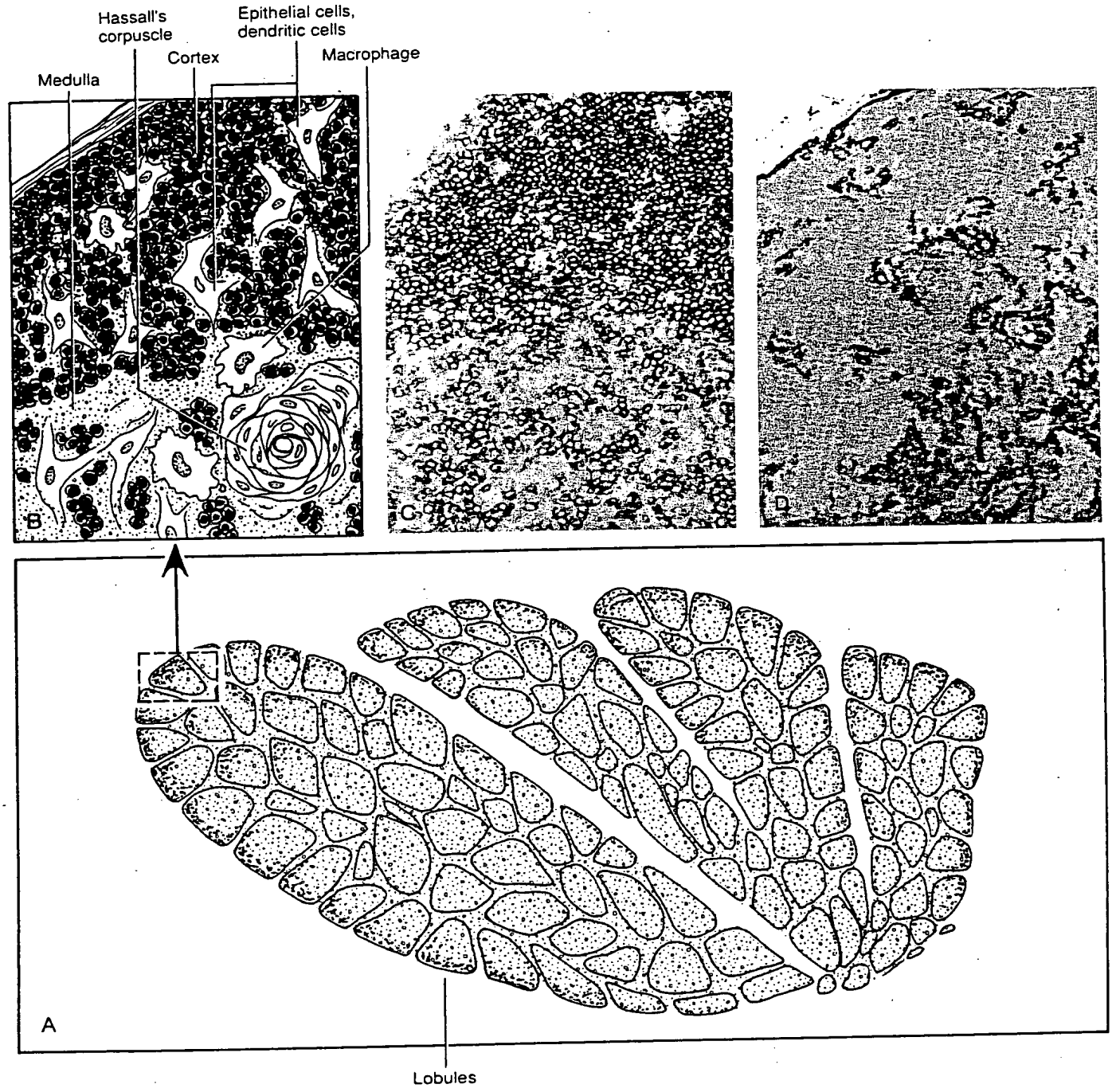

FIGURE 2 – 9. Morphology of the thymus.

A. *Schematic diagram of the thymus, illustrating two main lobes (the one on the right being subdivided into smaller lobes in this thymus), composed of multiple lobules.*

B. *Diagram of the edge of a lobule showing the cells of the cortex and medulla.*

C. *T lymphocytes in the cortex of the thymus, detected by an immunoperoxidase stain with an antibody specific for T cells. (Immunoperoxidase staining is described in Chapter 3; positive cells appear dark.) (Courtesy of Dr. G. S. Pinkus, Department of Pathology, Brigham and Women's Hospital, Boston.)*

D. *Epithelial cells scattered throughout the cortex and medulla, detected by an immunoperoxidase stain specific for keratin, an intracellular intermediate filament protein. (Courtesy of Dr. G. S. Pinkus, Department of Pathology, Brigham and Women's Hospital, Boston.)*

mice, about 50×10^6 immature cells enter the thymus each day and fewer than 1×10^6 mature cells leave. The thymus undergoes physiologic involution with aging, so that by puberty it is difficult to locate. Maturation and selection of T lymphocytes continue well into adult life, suggesting that either the remnants of the thymus that are present in adults are adequate for performing these functions or extrathymic T cell maturation can also occur. However, no such extrathymic sites of T cell development have been identified.

Lymph Nodes

Lymph nodes are small nodular aggregates of lymphoid tissue situated along lymphatic channels throughout the body. Epithelia, such as the skin and the mucosa of the gastrointestinal and respiratory tracts, as well as connective tissues and most organs have a lymphatic drainage. Antigens that enter through any of these portals end up in lymphatic vessels whose contents are "sampled" by lymph nodes for the presence of foreign substances. Each node is surrounded by a fibrous capsule that is pierced by numerous afferent lymphatics, which empty the lymph into a subcapsular sinus (Fig. 2-10). The node consists of an outer cortex in which there are aggregates of cells constituting the **follicles,** some of which contain central areas called **germinal centers** that stain lightly with commonly used histologic stains. The inner medulla contains less dense lymphocytes and mononuclear phagocytes scattered among lymphatic and vascular sinusoids. Lymphocytes and accessory cells are often found in close proximity but do not form intercellular junctions, which is probably important for maintaining their ability to migrate and recirculate between the lymph, blood, and tissues. The lymph that enters the subcapsular sinus percolates through the cortex and medulla and exits via a single efferent lymphatic located in the hilum of the node. In addition, each node has a vascular supply with afferent and efferent vessels at the hilum.

Different classes of lymphocytes and non-lymphoid accessory cells are sequestered in particular areas of the node. Follicles without germinal centers, which are called **primary follicles,** contain predominantly mature, resting B lymphocytes that have apparently not been stimulated recently by antigens. The germinal centers, which develop in response to antigenic stimulation, contain numerous large lymphocytes with phenotypic characteristics of activated B cells. The germinal centers are believed to be one of the sites where B lymphocytes proliferate and differentiate into antibody-secreting cells in response to antigenic stimulation. It is estimated that the cell cycle time of germinal center B cells is as short as 6 hours. Since plasma cells are rare at these sites, it is possible that the terminal differentiation of B cells occurs outside the germinal centers. It is also believed that the activation of memory B cells, the production of antibodies with increased affinities for antigens, and the appearance of different classes of antibodies (described in Chapters 4 and 9) are three aspects of antigen-stimulated B cell differentiation that are initiated largely in germinal centers. Since these events usually require the participation of helper T lymphocytes, it is not surprising that germinal center development is T cell-dependent and is not seen in individuals congenitally deficient in T cells. In addition to lymphocytes, lymphoid follicles contain macrophages and scattered **dendritic cells** (also called interdigitating reticular cells) that function as accessory cells in immune responses. Also, in the germinal centers there are **follicular dendritic cells** that have long cytoplasmic processes, express large numbers of receptors for antibodies (Fc receptors) on their surfaces, and are different from the "dendritic cells" mentioned above. Follicular dendritic cells are believed to be important in capturing antigens complexed with preformed antibodies and, therefore, in the activation of memory B cells to generate secondary antibody responses.

The T lymphocytes are located predominantly in the interfollicular areas of the cortex and paracortical zones in the medulla (Fig. 2-10). Most of these are $CD4^+$ helper T cells, intermingled with relatively sparse $CD8^+$ cells. Some $CD4^+$ T cells are also scattered in germinal centers, where their role may be to help the proliferation and differentiation of antigen-stimulated B lymphocytes. The proximity of helper T cells and the B cells that are the recipients of T cell help is important because helper function is mediated largely by secreted cytokines, which act at short distances, close to the sites where they are produced (see Chapter 9). Dendritic (interdigitating reticular) cells are abundant in the T cell areas, which is consistent with their postulated role in presenting foreign antigens to T lymphocytes.

The medulla contains scattered lymphocytes, large numbers of macrophages and dendritic cells, and, in nodes draining sites of immunization, numerous plasma cells, all of which are interspersed with lymphatic channels.

The mechanisms responsible for the anatomic sequestration of different classes of lymphocytes in distinct areas of the node are unclear. One possibility is that compartmentalization is maintained by specific adhesions of different lymphocytes with stromal cells or extracellular matrix proteins. Although the anatomy of the immune response is poorly understood, it is likely that this cellular organization promotes interactions between the participating cell types and is critically important for the generation of immunity. Studies using labeled antigens indicate that a protein antigen that enters the lymph node in an unimmunized individual is trapped by macrophages and dendritic cells and largely degraded. Peptide fragments of the injected antigen, attached to the surfaces of accessory cells, stimulate helper T lymphocytes (see Chapter 6). The first wave of mitotic activity is seen in the T cell zones within 1 to 2 days after immunization. Proliferation of B cells follows, after which germinal centers develop and the B lympho-

(F)

IMMUNOBIOLOGY

THE IMMUNE SYSTEM IN HEALTH AND DISEASE

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Competitive binding assays are serological assays in which unknowns are detected and quantitated by their ability to inhibit the binding of a labeled known ligand to its specific antibody.

The **complement** system is a set of plasma proteins that act together to attack extracellular forms of pathogens. Complement can be activated spontaneously on certain pathogens or by antibody binding to the pathogen. The pathogen becomes coated with complement proteins that facilitate pathogen removal by phagocytes and may also kill the pathogen.

Complement receptors (CR) are cell-surface proteins on various cells which recognize and bind complement proteins that have bound a pathogen. Complement receptors on phagocytes allow them to identify pathogens coated with complement proteins for uptake and destruction.

The **complementarity determining regions (CDRs)** of immunological receptors are the parts of the receptor that make contact with specific ligand and determine its specificity. The CDRs are the most variable part of the receptor protein, giving receptors their diversity, and are carried on six loops at the distal end of the receptor's variable domains, three loops coming from each of the two variable domains of the receptor.

Some epitopes on a protein antigen are called **conformational** or **discontinuous epitopes** because they are formed from several separate regions in the primary sequence of a protein by protein folding. Antibodies that bind conformational epitopes only bind native, folded proteins.

Congenic strains of mice are genetically identical at all loci except one. Each strain is generated by the repetitive backcrossing of mice carrying the desired trait onto a strain that provides the genetic background for the set of congenic strains. The most important congenic strains in immunology are the **congenic resistant strains**, developed by George Snell, that differ from each other at the MHC.

Constant regions: see **C regions**.

Contact hypersensitivity is a form of delayed-type hypersensitivity in which T cells respond to antigens that are introduced by contact with the skin. Poison ivy is a contact sensitivity reaction due to T-cell responses to the chemical antigen pentadecacatechol in poison ivy leaves.

Continuous or **linear epitopes** are antigenic determinants on proteins that are contiguous in the protein sequence and therefore do not require protein folding for antibody to bind. See also **conformational** or **discontinuous epitopes**.

A **convertase** is an enzymatic activity that converts a complement protein into its reactive form by cleaving it. Generation of the C3/C5 convertase is the pivotal event in complement activation.

The **Coombs test** is a test for antibody binding to red blood cells. Red blood cells that are coated with antibody are agglutinated if they are exposed to an anti-immunoglobulin antibody. The Coombs test is important in detecting the non-agglutinating antibodies to red blood cells produced in Rh incompatibility.

A **co-receptor** is a cell-surface protein that increases the sensitivity of the antigen receptor to antigen by binding to associated ligands and participating in signaling for activation. CD4 and CD8 are MHC-binding co-receptors on T cells, while CD19 is part of a complex that makes up a co-receptor on B cells.

Corticosteroids are steroids related to those produced in the adrenal cortex, such as cortisone. Corticosteroids can kill lymphocytes, especially developing thymocytes, inducing apoptotic cell death. They are useful anti-inflammatory and immunosuppressive agents.

The proliferation of lymphocytes requires both antigen binding and the receipt of a **co-stimulatory signal**, usually delivered by a cell-surface molecule on the cell presenting antigen. For T cells, the co-stimulatory signals are B7 and B7.2, related molecules that act on the T-cell surface molecules CD28 and CTLA-4. For B cells, CD40 ligand acting on CD40 serves an analogous role.

Cowpox is the common name of the disease produced by vaccinia virus, used by Edward Jenner in the successful vaccination against smallpox, which is caused by the related variola virus.

C-reactive protein is an acute phase protein that binds to phosphatidylcholine which is a constituent of the C-polysaccharide of the bacterium *Streptococcus pneumoniae*, hence its name. Many other bacteria also have surface phosphatidylcholine that is accessible to C-reactive protein, so C-reactive protein can bind many different bacteria and opsonize them for ready uptake by phagocytes.

C regions or **constant regions** of antibody molecules and T-cell receptors are made up of one or more **C domains**, each encoded in a single exon. As only a single gene encodes each C region, it has the same structure in all antibodies or T-cell receptors in which it is expressed.

Cross-matching is used in blood typing and histocompatibility testing to determine whether donor and recipient have antibodies against each other's cells that might interfere with successful transfusion or grafting.

A **cross-reaction** is the binding of antibody to an antigen not used to elicit that antibody. Thus, if antibody raised against antigen A also binds antigen B, it is said to cross-react with antigen B. The term is used generically to describe the reactivity of antibodies or T cells with more than the eliciting antigen.

CTLA-4 is a cell-surface molecule found on activated T cells; it is a receptor for **B7** and **B7.2**.

Cutaneous T-cell lymphoma is a malignant growth of T cells that home to the skin.

Cyclophosphamide is an alkylating agent that is used as an immunosuppressive drug. It acts by killing rapidly dividing cells including lymphocytes proliferating in response to antigen.

Cyclosporin A is a potent immunosuppressive drug that inhibits signaling from the T-cell receptor, preventing T-cell activation and effector function. It binds to cyclophilin, and this complex binds to and inactivates the serine/threonine phosphatase **calcineurin**.

Cytokines are proteins made by cells that affect the behavior of other cells. Cytokines made by lymphocytes are often called **lymphokines** or **interleukins** (abbreviated IL), but the generic term cytokine is used in this book and most of the literature. Cytokines act on specific cytokine receptors on the cells they affect. Cytokines and their receptors are listed in Appendix II.

Cytokine receptors are cellular receptors for cytokines. Binding of the cytokine to the cytokine receptor induces new activities in the cell, such as growth, differentiation, or death. Cytokine receptors are listed in Appendix II.

Cytotoxic T cells are T cells that can kill other cells. Most cytotoxic T cells are MHC class I-restricted CD8 T cells, but CD4 T cells can also kill in some cases. Cytotoxic T cells are important in host defense against cytosolic pathogens.

Cytotoxins are proteins made by cytotoxic T cells that participate in the destruction of target cells. **Perforins** and **granzymes** or **fragmentins** are the major defined cytotoxins.

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